

Synthetic and Biosynthetic Studies of  
Some Fungal Metabolites

by

Charles Rupert McIntyre, B.Sc.

A thesis presented for the  
degree of Doctor of Philosophy

University of Edinburgh



March 1985

Dedication

To my parents, in recognition of all  
they have done for me



Declaration

I declare that this thesis is my own composition and that the work of which it is an account is original. Wherever the results or ideas of previous workers are cited, this is specifically indicated by reference. The research was undertaken in the Chemistry Department of the University of Edinburgh, under the supervision of Dr. Thomas J. Simpson, after the 1st October 1980, the date of my admission as a postgraduate student. No part of this thesis has been previously submitted towards a higher degree or qualification although some of the results have already been published elsewhere.

### Acknowledgements

I wish to record my great indebtedness to my research supervisor, Dr. Thomas J. Simpson, for suggesting the topics of research and for his invaluable support and encouragement throughout this project. I am also grateful to the academic and technical staff of the Chemistry Department, University of Edinburgh, without many of whom this work would not have been possible. Particular recognition is due to: Dr. Ian H. Sadler, Dr. David Reed, Dr. Alan S.F. Boyd, John R.A. Millar, Laurence H. Bell and Fiona E. Scott for n.m.r. spectra, to David Thomas, Dr. Elaine A. Abbott and Alan T. Taylor for mass spectrometry, and to John Grunbaum for microanalyses. Suzan M. Kasperek deserves special mention for her immaculate execution of the extensive microbiological work.

Somewhat further afield, thanks are also due to: Dr. John S.E. Holker and Dr. Eugene O'Brien, both of the University of Liverpool, for providing isotopically-labelled orsellinates, to Dr. John C. Vederas, Laird A. Trimble and Richard N. Moore, all of the University of Alberta, for supplying  $^{18}\text{O}$ -labelled precursors and for performing some difficult n.m.r. spectroscopy, and to Lederle Laboratories, New York, for providing a sample of LL-D253 $\alpha$ .

To all of the above and to my laboratory friends and colleagues, I extend my sincere thanks. The award of an S.E.R.C. studentship is also gratefully acknowledged.

ABSTRACT

This thesis is divided into five chapters, the first two of which are introductory. In Chapter 1 the secondary metabolism of fungi is briefly discussed with special reference to the polyketide pathway. Chapter 2 comprises a short review of the isotopic tracer methods important in biosynthetic studies, concentrating on the use of nuclear magnetic resonance techniques.

Chapter 3 concerns LL-D253 $\alpha$ , a 4-chromanone metabolite of *Phoma pigmentivora*. Spectroscopic evidence has led to a revision of the original structure; both this and the new structure have been synthesised. [2-<sup>14</sup>C]-, [1-<sup>13</sup>C]-, [2-<sup>13</sup>C]-, [1,2-<sup>13</sup>C<sub>2</sub>], [1-<sup>13</sup>C, <sup>2</sup>H<sub>3</sub>]-, [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]- and [<sup>2</sup>H<sub>3</sub>]acetates, [1-<sup>14</sup>C]glycollate, [U-<sup>14</sup>C]oxalate, [5-<sup>14</sup>C]-mevalonate and <sup>18</sup>O<sub>2</sub> gas have all been administered to *P. pigmentivora*. The results indicate a polyketide biogenesis for LL-D253 $\alpha$  and a two-chain pathway has been proposed to account for the structure. A spiro-cyclopropyl intermediate formed by aryl participation is postulated to explain the unusual scrambling of carbon and hydrogen label observed in the 2-hydroxyethyl side-chain. Possible mechanisms for formation of the pyranone ring are discussed.

Chapter 4 concerns meroterpenoid metabolites of *Aspergillus* species. The incorporations of <sup>18</sup>O<sub>2</sub> gas and [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate into andibenin B and andilesin A, both known triprenyl-phenols of *A. variegator*, suggest possible

pathways for the formation of the spiro-lactone moiety of the andibenins. Incorporation of acetate oxygen into andilesin A was too low to distinguish between possible orsellinate and deoxyorsellinate intermediates.

The biosynthesis of terretonin by *A. terreus* has been investigated. Assignment of the  $^{13}\text{C}$  n.m.r. spectrum is described in detail. Incorporations of  $[1,2-^{13}\text{C}_2]\text{acetate}$  and  $[\text{carboxyl}, 2-^{14}\text{C}]$ - and  $[3\text{-methyl}, ^2\text{H}_3]\text{-3,5-dimethyl-orsellinates}$  indicate a triprenyl-phenol biogenesis, contrary to previous suggestions.

In Chapter 5 the incorporations of  $^{18}\text{O}_2$  gas into tajixanthone and shamixanthone, metabolites of *A. variegator*, are discussed. The results are consistent with earlier observations indicating the occurrence of free rotation of the symmetrical element of a benzophenone intermediate. The principal mode of xanthone ring closure is demonstrated. Evidence that all the atmospherically-derived oxygen atoms are independently introduced is presented and a pathway *via* an anthraquinone intermediate is suggested.

Contents

Declaration	iii
Acknowledgements	iv
Abstract	v
Contents	vii

<u>Chapter 1</u>	<u>Introduction: Biosynthesis of Fungal Metabolites</u>	1
Section	1.1 Secondary Metabolism	2
	1.2 Fungal Metabolites	2
	1.3 The Polyketide Pathway	3
	1.4 The Terpenoid Pathway	7
	1.5 References	9
 Chapter 2	 <u>Introduction: The Methodology of Tracer Studies</u>	 11
Section	2.1 Preamble	12
	2.2 The Design and Interpretation of Tracer Experiments	12
	2.3 Radio-Isotopic Labelling	14
	2.4 Use of N.M.R. Spectroscopy in Tracer Studies	16
	2.4.1 Use in Tracing Carbon	16
	2.4.1.1 $^{13}\text{C}$ N.m.r. Spectroscopy	16
	2.4.1.2 Single $^{13}\text{C}$ -Labelling	17
	2.4.1.3 Double $^{13}\text{C}$ -labelling	19
	2.4.1.4 $^{13}\text{C}$ -Depleted Materials	21
	2.4.1.5 Quantitative $^{13}\text{C}$ Measurements	22
	2.4.2 Use in Tracing Hydrogen	24
	2.4.2.1 Hydrogen Isotopes	24
	2.4.2.2 Protium	25

2.4.2.3	Deuterium	26
2.4.2.4	Tritium	30
2.4.3	Use in Tracing Oxygen	31
2.4.3.1	$^{17}\text{O}$ -Labelling	31
2.4.3.2	$^{18}\text{O}$ -Labelling	32
2.5	References	34
Chapter 3	<u>Structural Revision, Synthesis and</u> <u>Biosynthesis of LL-D253<math>\alpha</math></u>	38
Section 3.1	Background Information: Structure	39
3.2	Structural Revision and $^{13}\text{C}$ Spectral Assignment of LL-D253 $\alpha$	42
3.3	Synthesis of LL-D253 $\alpha$ and Two of its Isomers	49
3.3.1	Synthesis of LL-D253 $\alpha$	49
3.3.2	Synthesis of Two Isomers of LL-D253 $\alpha$	57
3.3.3	Identification of the Two Isomers	60
3.4	Background Information - Biosynthesis	62
3.5	Biosynthetic Studies of LL-D253 $\alpha$	65
3.5.1	Preliminary Investigations	65
3.5.2	Incorporation of $^{13}\text{C}$ -Labelled Acetates	66
3.5.3	Investigation of Possible Sources of the Hydroxyethyl Side-Chain	71
3.5.4	Incorporation of $[1-^{13}\text{C}, ^2\text{H}_3]$ - acetate	73
3.5.5	Mechanism of Pyranone Ring- Formation - Incorporation of $[1-^{13}\text{C}, ^{18}\text{O}_2]$ acetate	76
3.5.6	Mechanism of Skeletal Formation - Incorporation of $^{18}\text{O}_2$ Gas	80
3.5.7	Possible Future Experiments	84
3.6	Experimental	86
3.6.1	General Procedures and Instrumentation	86
3.6.2	Synthetic Experimental	87
3.6.3	Biosynthetic & Related Experimental	122
3.7	References	138

Chapter 4	<u>Biosynthetic Studies on Some <i>Aspergillus</i></u>	144
	<u>Triprenyl-Phenols</u>	
Section 4.1	Background Information	145
4.2	$^{18}\text{O}$ -Labelling of Andibenin B and Andilesin A	148
4.2.1	Object of Experiments	148
4.2.2	Preliminary Investigations	149
4.2.3	Incorporation of $^{18}\text{O}$ -Labelled Precursors	150
4.2.4	Results: Andibenin B	153
4.2.5	Results: Andilesin A	157
4.3	Biosynthetic Studies on Terretonin	159
4.3.1	Preamble: Object of Experiments	159
4.3.2	Production of Terretonin	160
4.3.3	Assignment of the $^{13}\text{C}$ N.M.R. Spectrum	160
4.3.4	Incorporation of $[2-^{14}\text{C}]$ acetate	171
4.3.5	Incorporation of $^{13}\text{C}$ -Labelled Acetates and Methionine	171
4.3.6	Incorporation of Labelled Orsellinates	174
4.3.7	Conclusions	176
4.4	Experimental	179
4.4.1	Andibenin B/Andilesin A	179
4.4.2	Terretonin	183
4.5	References	189
Chapter 5	<u>Biosynthetic Studies of Tajixanthone and Shamixanthone</u>	193
Section 5.1	Background Information	194
5.2	Origin of the Oxygen Atoms of Tajixanthone & Shamixanthone	197
5.2.1	Object of Experiment	197
5.2.2	Incorporation of $^{18}\text{O}_2$	198
5.2.3	Results & Conclusions	200

5.3	Mechanism of the Ring-Cleavage	202
5.3.1	Background to Experiment	202
5.3.2	Design of $^{16}\text{O}_2$ - $^{18}\text{O}_2$ Mixed Atmosphere Experiment	204
5.3.3	Results & Conclusions	207
5.4	Experimental	210
5.5	References	214
Appendix:	Apparatus for the Measured Utilisation of Molecular Oxygen by Fungal Cultures	217
Publications		220
Lecture Courses		



## Chapter 1

### Introduction: Biosynthesis of Fungal Metabolites

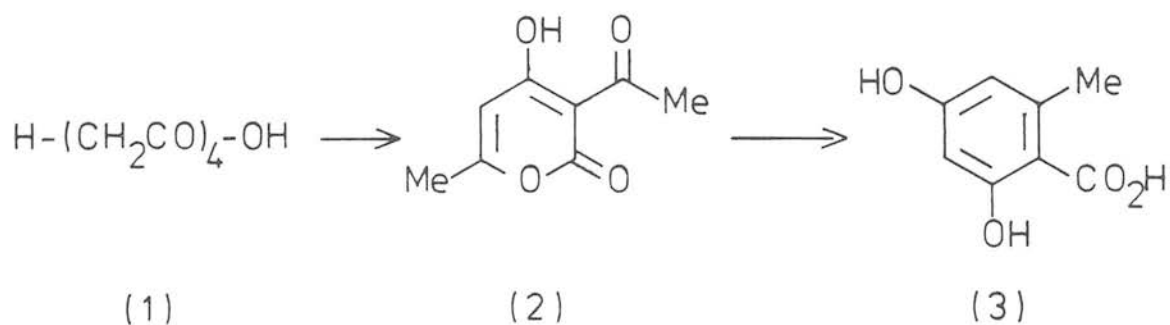
## 1. Introduction: Biosynthesis of Fungal Metabolites

### 1.1 Secondary Metabolism

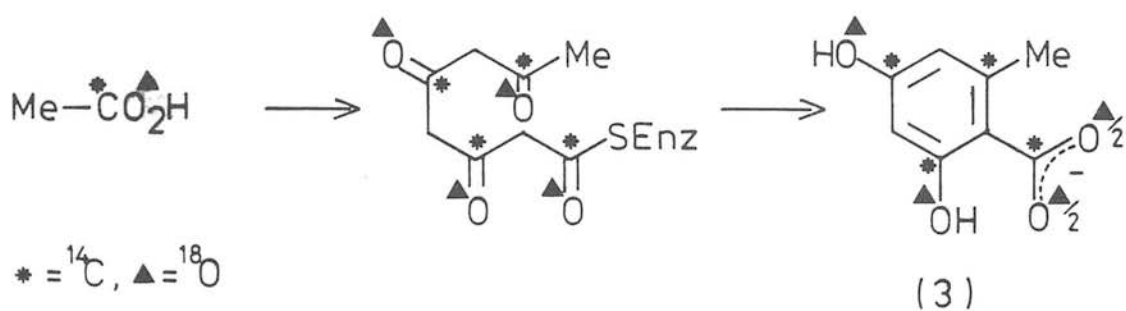
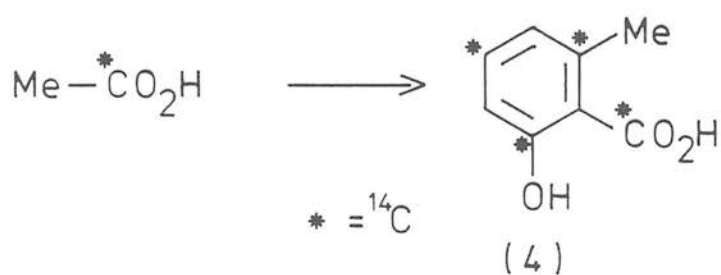
Many micro-organisms, plants and lower animals produce, sometimes in large amounts, compounds which are not essential to the maintenance of life: these are the "secondary metabolites" or "natural products".<sup>1</sup> The terms may also be extended to cover a metabolite, normally regarded as essential or primary, which is being produced in inexplicably large quantities, *e.g.* citric acid from *Aspergillus niger*. Although many of these compounds do have biological functions in communication and defence<sup>2</sup>, the majority are of no known use to the producing organism. In spite of their structural diversity and individually-limited taxonomic distribution, it has been possible to characterise most of them, at least tentatively, as the products of one or more of a very few, albeit highly flexible, metabolic pathways.

### 1.2 Fungal Metabolites

Fungi are convenient organisms for the study of secondary metabolism. They produce a vast range of compounds<sup>3,4</sup>, many of which are of intrinsic interest due to their biological activities. Yields of metabolites are often high and isolation procedures are generally simple. As the incorporation of precursor compounds is usually much more efficient in micro-organisms than in higher species, the less sensitive but more informative tracer techniques,



Scheme 1



Scheme 2

notably n.m.r. spectroscopy (see Section 2.4), can be used.

Two secondary pathways are of particular importance in fungi and are relevant to the work to be described later. These are the polyketide and terpenoid pathways.

### 1.3 The Polyketide Pathway

Many accounts of polyketide biosynthesis have been published<sup>5-9</sup>. The pathway was first envisaged and named by Collie (1907)<sup>10</sup> who pointed out that the " $\text{CH}_2\text{C}=\text{O}$ " group or "keten" group could be easily condensed into polyacetic acids and thence into the kind of compounds found in plants, *e.g.* tetra-acetic acid (1) allegedly lost water to form "dehydracetic acid" (2) which, on treatment with alkali, gave orcinol-carboxylic acid (orsellinic acid) (3) (Scheme 1). He correctly surmised that fatty-acids and terpenes also arose from acetic acid but he included carbohydrates in this scheme. (This is generally incorrect: acetate is not a usual precursor of carbohydrates). Unfortunately, Collie's ideas went largely ignored until 1953 when Birch<sup>11</sup> independently formulated the "acetate hypothesis" from consideration of the structures of many natural phenols. The hypothesis was initially refused publication until its ability to predict and correct structures was demonstrated<sup>12</sup>. More direct evidence for its validity came with the incorporation of  $[1-^{14}\text{C}]$ acetic acid into 6-methylsalicylic acid (4), a common microbial metabolite, with the required alternate

labelling pattern<sup>13</sup>. An important observation<sup>14</sup> in further support was made in 1959 when  $[1-^{14}\text{C}, ^{18}\text{O}]$ acetate was shown to incorporate into orsellinic acid (3) with a retention of labels which indicated that the ring oxygens were derived from acetate and the carboxyl oxygens of the metabolite were derived half from acetate and half by hydrolysis (Scheme 2).

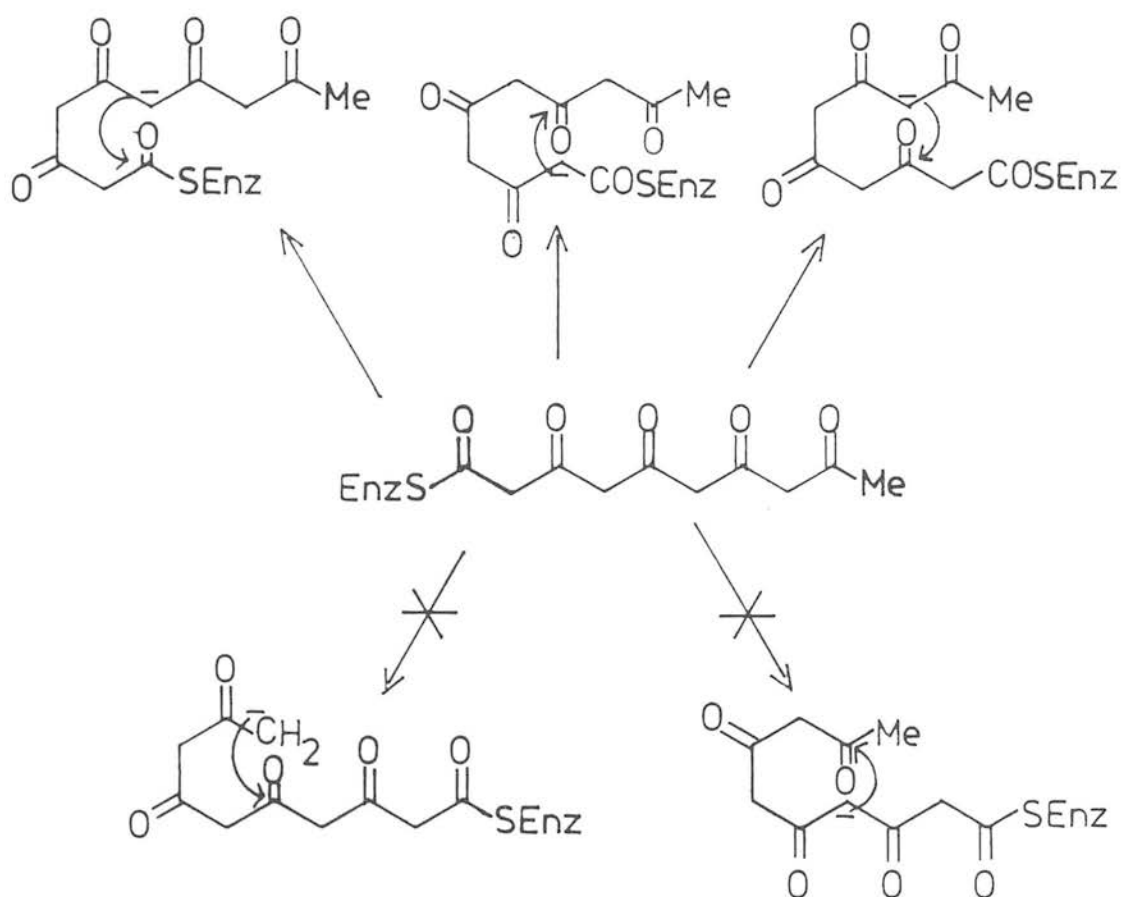
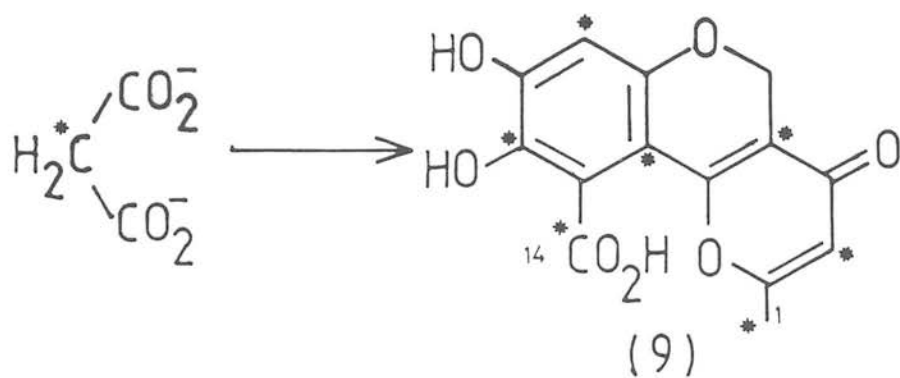
Further biosynthetic studies, mostly using micro-organisms, have established a large body of evidence on polyketide biogenesis. Polyketides generally comprise structures which are formally derived from poly- $\beta$ -ketomethylene chains,  $-\text{[CHRCO]}_n-$ . The number of units,  $n$ , usually ranges between 4 and 20 and the unit itself is usually, but not always, acetate ( $\text{R}=\text{H}$ ). Aldol and Claisen type condensations provide the basic skeletal types.

The poly- $\beta$ -ketomethylene chain is built from a "starter" unit - an acyl co-enzyme A - and further units of (usually) malonyl co-enzyme A which are added to the chain by successive condensations with concomitant decarboxylations. (Fatty acid biosynthesis is a special case where each ketomethylene unit is fully reduced after each condensation.) The assembly of acyl and malonyl units is mediated by an enzyme system which takes up these precursor units and releases a stabilized polyketide: all intermediate species are enzyme-bound. An illustration is provided by the closely studied biosynthesis of 6-methylsalicylic acid (6-MSA) (Scheme 3).



In *Penicillium patulum*, 6-MSA is synthesised by a multienzyme complex (5) containing, *inter alia*, two types of thiol group: one is a "carrier site" and the other is a "condensing site". The acyl residues are transferred onto the thiol sites by means of specific transacylases and the proximity of the sites allows the controlled acylation of the residue of the carrier site by that on the condensing site. This intermediate product is then transferred to the condensing site and another unit of malonate is transferred to the carrier site. The process is repeated, with one intermediate reduction step, until an enzyme-bound tetraketide (6) is formed. This is stabilised by cyclisation (aldol reaction) and aromatisation (by enolisation) and split from the enzyme complex to give 6-MSA (4) directly. In the absence of the reducing co-factor, NADPH, the "derailment product", tri-acetic lactone (7), is formed instead.

It is important to note that this enzyme complex, in common with most fungal polyketide synthetases, will only accept acetyl and malonyl residues for condensation. Acetoacetyl residues, for instance, are not taken up even though they are implicated in enzyme-bound form (8). As malonate usually arises from the direct carboxylation of acetate, and this carboxyl group is subsequently lost on condensation, many fungal polyketides can be described as "wholly acetate-derived". (This is not always the case: other starter units are often acceptable,



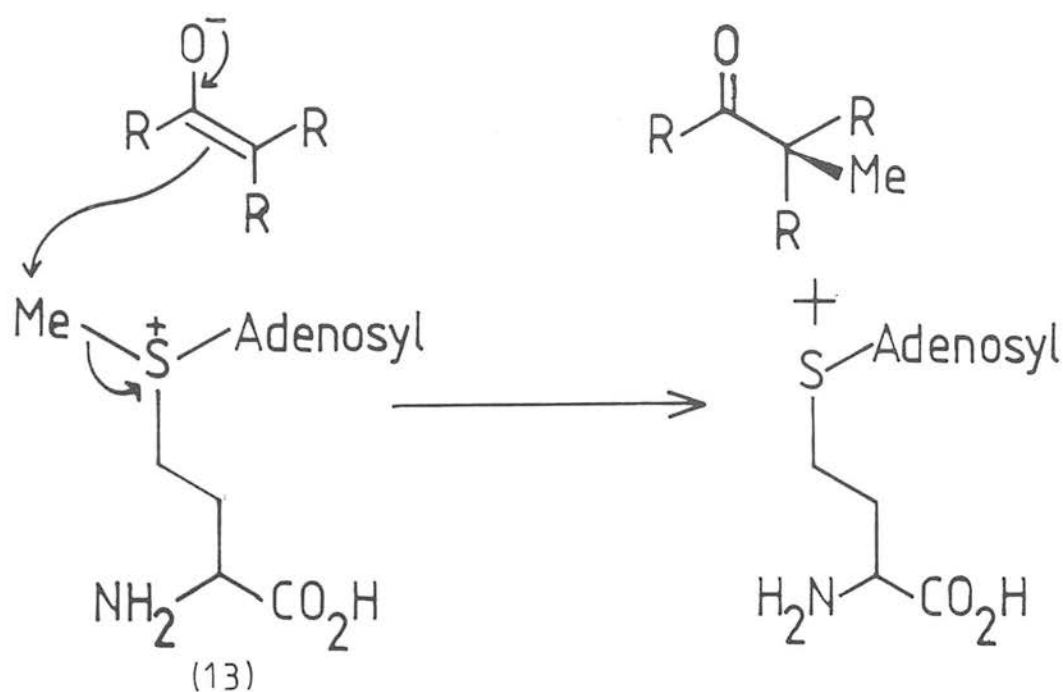
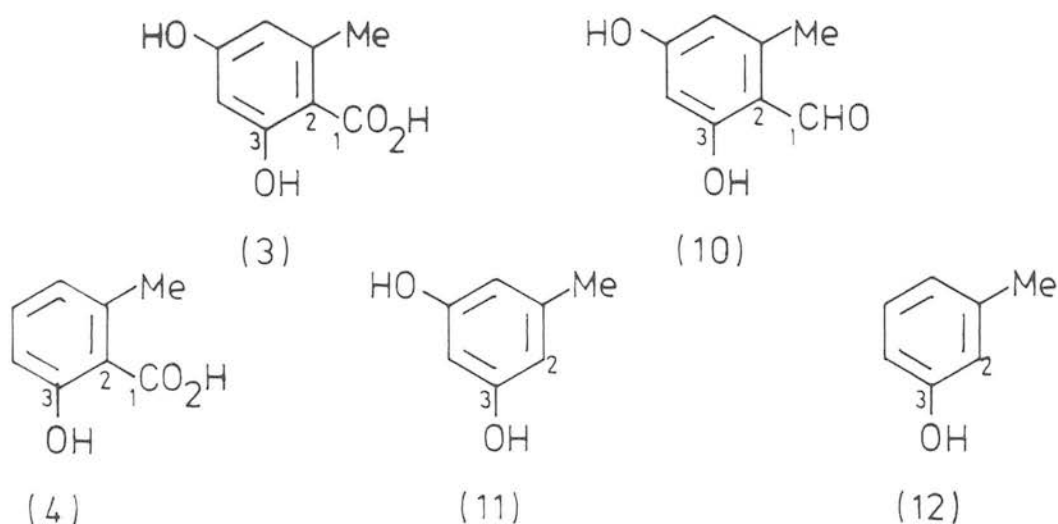
Scheme 4 Possible cyclisations of a pentaketide chain



especially in plant systems, and different propagation units, such as methylmalonate, are often employed by the filamentous Actinomycetes bacteria.)

A significant metabolic pool of malonate appears to exist in some cases; hence, when labelled acetate is used as a tracer, somewhat heavier labelling may be observed in the acetyl Co-A derived starter unit than elsewhere. Conversely, malonate may label the propagation units of a polyketide more efficiently. These "starter effects" are not seen if sufficiently rapid equilibration between malonate and acetate occurs but they can give useful information. For example, the incorporation of [2-<sup>14</sup>C]malonate into citromycetin (9) gave a non-uniform distribution of activity. C-1 and C-14 each carried less <sup>14</sup>C than the remaining labelled carbons, pointing to a biosynthesis from two separate polyketide chains<sup>15</sup>.

Examination of polyketide structures with a view to their biogenesis reveals two general "rules". Firstly, the cyclisation of the poly- $\beta$ -ketomethylene chain almost never occurs to give a shorter uncyclised residue from the methyl end of the chain than from the carboxyl end (Scheme 4). Secondly, although acetate oxygen may be lost from almost any position, it is always retained at the position derived from the  $\beta$ -carbonyl group when the methylene group  $\alpha$  to the terminal carboxyl group takes part in a cyclisation. Thus, for example, orsellinic



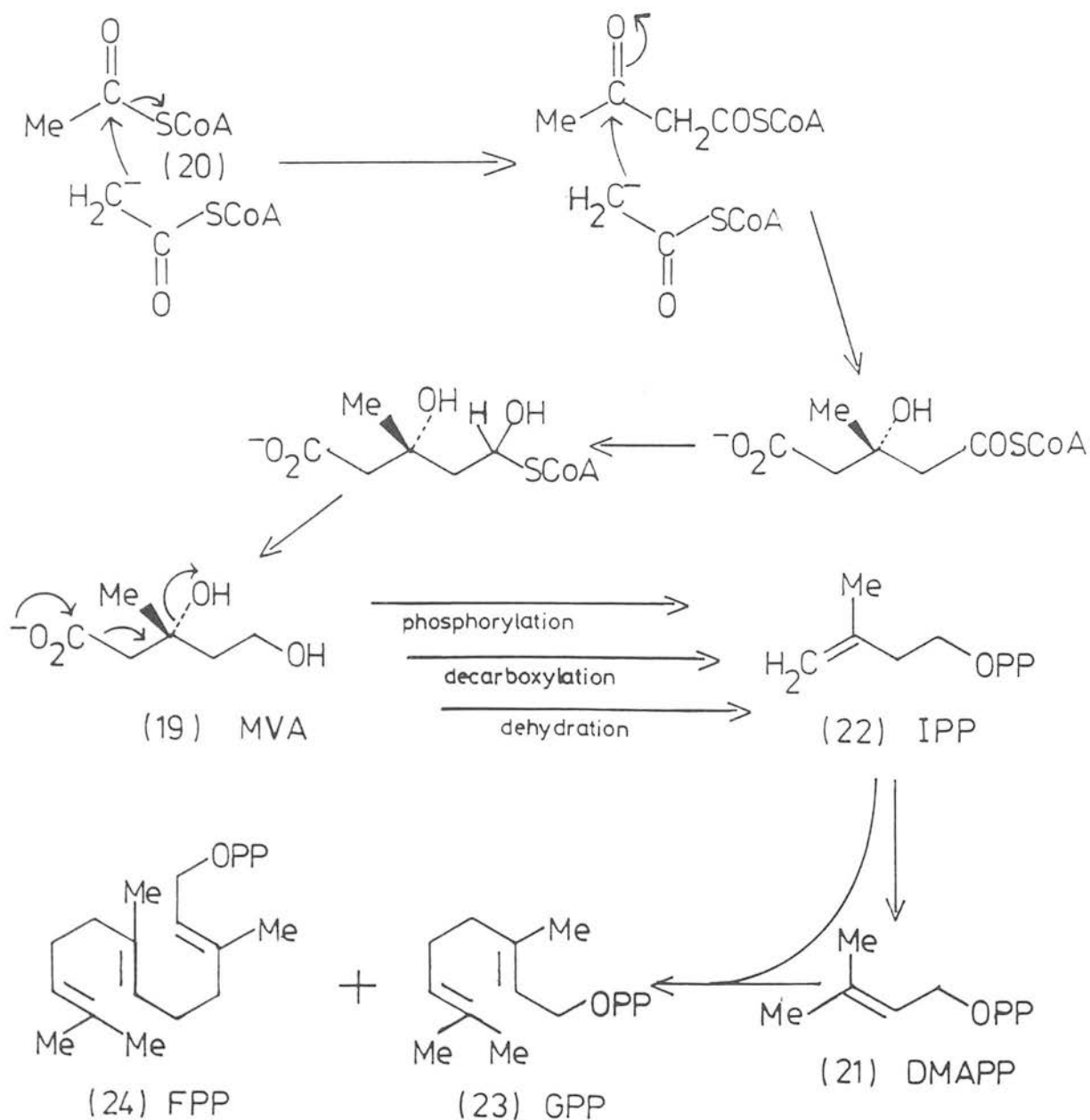
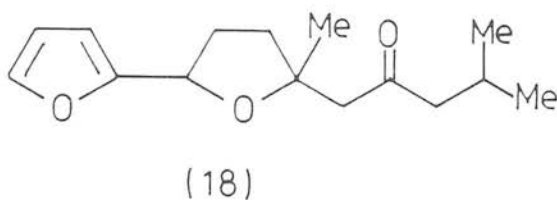
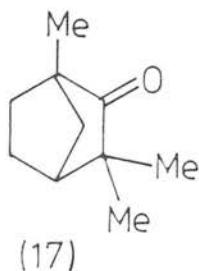
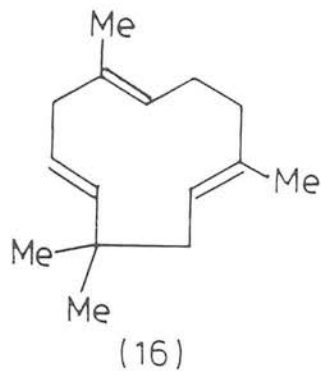
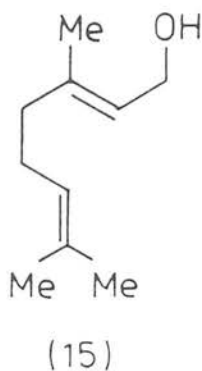
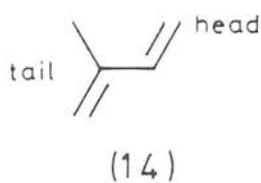
Scheme 5 C-methylation by S-adenosylmethionine

acid (3), orsellinaldehyde (10), 6-MSA (4), orcinol (11) and *m*-cresol (12) are all fungal tetraketides cyclised in this way<sup>3</sup> but no comparable metabolite lacking oxygen at C-3 is known.

A wide range of other reactions can either be interpolated with the basic chain assembly process or can act on the stable free polyketide. Oxidations, reductions, decarboxylations, phenolic couplings, ring cleavages, and halogenations are all observed. Carbon alkylation, notably by methyl and dimethylallyl residues, can occur at the enzyme-bound stage but larger prenyl groups seem only to alkylate free polyketides. In the case of methylation, the donor is *S*-adenosylmethionine (13), a component of the "C<sub>1</sub> pool" of primary metabolism<sup>16</sup>. *C*-Methylation is usually mediated by a transmethyrase component of the multi-enzyme-complex, occurring by electrophilic attack at the enolisable methylene positions (Scheme 5). The prenylating agents, dimethylallyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) arise from the terpenoid route.

#### 1.4 The Terpenoid Pathway

Comprehensive accounts of terpenoid biosynthesis are available<sup>17-20</sup>. The elucidation of this pathway leading to, *inter-alia*, prenyl residues followed from the early observation that many natural products - the terpenes - could formally be derived by the head to tail joining



Scheme 6 Biosynthesis and fate of MVA

of isoprene (14) units; *e.g.* geraniol (15) and humulene (16). In 1953 Ruzicka expressed<sup>21</sup> the "biogenetic isoprene rule"; *i.e.* that some biological C<sub>5</sub> unit was polymerised and the resulting structure could then be modified enzymatically, in order to accommodate irregular terpenes such as fenchone (17) and ipomearone (18). The actual identity of the biogenetic isoprene equivalent was found to be mevalonic acid (MVA) in 1956.

The biosynthetic pathway to and from MVA (19) has since been investigated in great detail, notably by Bloch, Lynen and Cornforth, and is summarised in Scheme 6. Again acetyl Co-A (20) is the prime precursor. Dimethylallyl pyrophosphate (DMAPP) (21) and isopentenyl pyrophosphate (IPP) (22), the two initial, isomeric products, act, respectively, as the starter units and chain extending units of isoprenoid biosynthesis yielding GPP (23) and FPP (24).

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## Chapter 2

Introduction: The Methodology of Tracer Studies



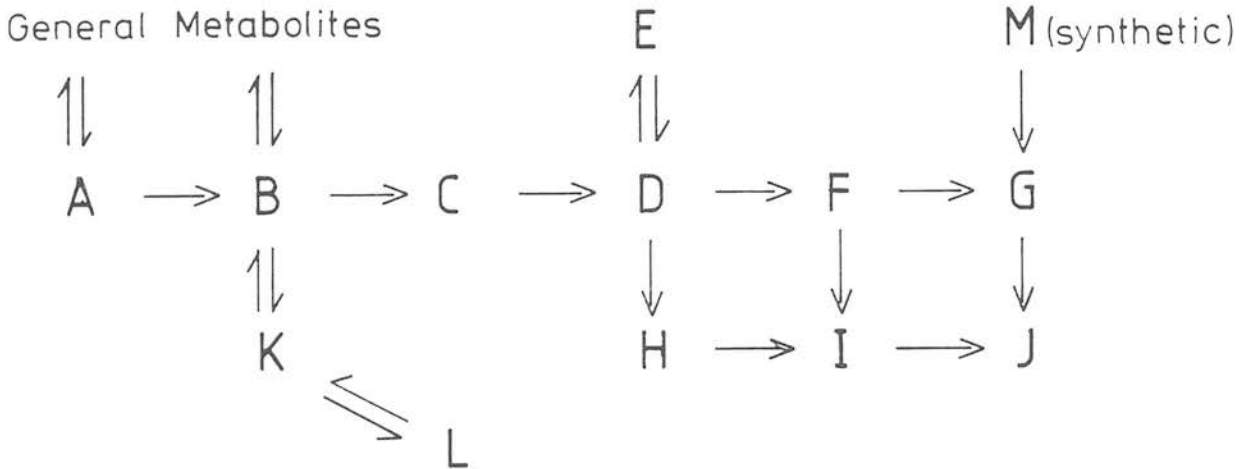
## 2. Introduction: The Methodology of Tracer Studies

### 2.1 Preamble

The association of natural products and chemistry is a close one: indeed, the early nineteenth-century efforts to isolate and characterise some of the more lethal plant products were directly responsible for the rapid development of organic chemistry. One result of this is the predominance of "chemical techniques" in the study of secondary metabolism, although the application of some of the powerful, if experimentally demanding, methods of biochemistry has also yielded valuable information, particularly on the earlier stages of secondary pathways. Although this section will concentrate on the use of nuclear magnetic resonance (n.m.r.) spectroscopy in tracer studies, brief discussions of experimental principles and of radio-isotopic tracing are also included.

### 2.2 The Design and Interpretation of Tracer Experiments

This subject has been discussed in several texts<sup>1-3</sup>. In the endeavour to identify the precursors and intermediates of a biosynthetic route, the usual technique is to attempt to assess the degree of utilisation of likely compounds. However, low or negligible levels of incorporation, even of true precursors, are often observed. Simple compounds of general metabolic importance often show low incorporations into the metabolite of interest because of the diversity of routes open to them. In the



J is the metabolite of interest.

A and B are precursors. (The term "prime-precursor" has been coined<sup>1</sup> for the species furthest into the secondary route which is also involved in general metabolism - in this case B.)

C and D are obligatory intermediates.

F, G, H, and I are non-obligatory intermediates.

K and L are not intermediates but may label J non-specifically.

E is a "shunt metabolite"

M is not a natural intermediate but is utilisable by the organism.

Scheme 1

usual case of experiments employing intact cells, structurally complex or highly polar species may find the cell membrane insufficiently permeable and never reach the site of biosynthesis. Hence, a negative result does not generally permit firm conclusions.

The isolation and incorporation of other metabolites can be a useful method of obtaining clues to the later stages of a pathway but, again, the results must be interpreted with caution (see Scheme 1). Thus a compound may be isolated which would be a very reasonable intermediate (L, in Scheme) and which shows incorporation into the final metabolite (J) when an isotopically labelled sample is administered to the producing organism. However, unless it can be shown that label has been incorporated "specifically" (*i.e.* from a known position in the putative intermediate to a corresponding position in the metabolite of interest) the possibility remains that degradation, to a common earlier intermediate or precursor, followed by reincorporation of this degradation product, has taken place. Some of the work on the aflatoxin series has been criticized on this account.

Even if specific incorporation can be demonstrated, the supposed intermediate still may not necessarily lie on the main pathway, but may be formed, reversibly, as a "shunt metabolite" (E). An entirely foreign substrate (M) may also show efficient and specific incorporation.

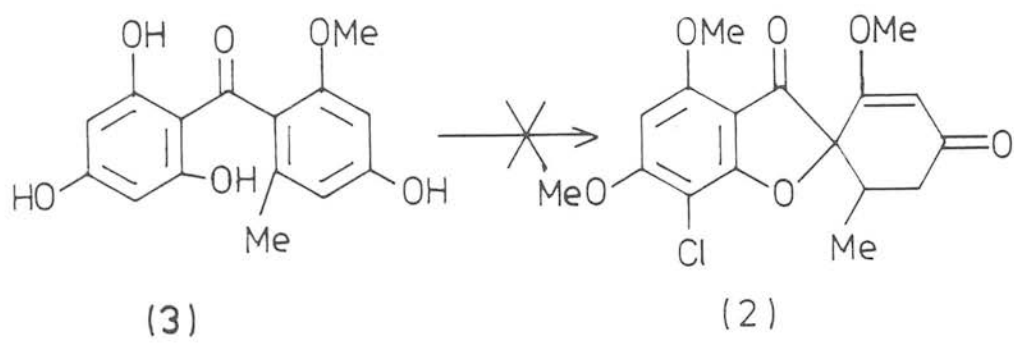
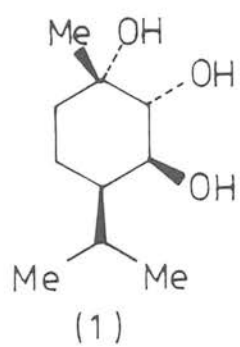
A further complicating factor is the often-observed relative lack of specificity of the enzymes of secondary metabolism, giving rise to what has been termed a "metabolic grid"<sup>1</sup> (D, F, G, H, I, J in Scheme). As drawn there are three possible routes from D to J, thus F, G, H and I are all possible, but not obligatory, intermediates.

### 2.3 Radio-Isotopic Labelling

Until the ascendancy of n.m.r. techniques, radio-labelling<sup>2,3</sup>, usually with the soft  $\beta$ -emitters  $^{14}\text{C}$  and  $^3\text{H}$  (tritium), was the single most powerful method of biosynthetic investigation. The major advantage of these radionuclides is their negligible natural abundance which, when coupled with the low limits of detection of modern liquid scintillation<sup>4</sup> counters, allows very low levels of precursor incorporation to be accurately determined. Only very small quantities of labelled precursors are required providing the specific activity is sufficiently high; thus the usual assumption that the tracer does not itself affect the metabolism under study is likely to be valid.

An alternative detection technique, that of autoradiography, is useful for identifying intermediates on a pathway and for testing the involvement of potential precursors in small scale experiments<sup>5</sup>.

The main drawbacks of radiolabelling are the need to obtain a radiochemically pure sample (sometimes very difficult) and the problem of establishing specificity of



labelling. This latter usually requires experimentally demanding and sometimes impractical degradations, especially where the label originates from a simple precursor such as acetate and is distributed throughout the molecule. More complex intermediates can be labelled at two sites with both tritium and  $^{14}\text{C}$  in a known ratio and administered to an organism: the isolation of the product with the same  $^3\text{H}:^{14}\text{C}$  ratio is interpreted as good evidence for intact incorporation.

$^3\text{H}$ ,  $^{14}\text{C}$  double labelling is also used to measure the retention of hydrogen atoms from precursor molecules. Thus *Fusicoccum amygdali* fed  $[5\text{-}^3\text{H}, 2\text{-}^{14}\text{C}]\text{mevalonate}$  produced  $1\alpha$ ,  $2\alpha$ ,  $3\beta$ -trihydroxy-*p*-menthane (1) with a  $^3\text{H}:^{14}\text{C}$  ratio corresponding to the incorporation of four mevalonate-derived hydrogen atoms per molecule - one more than the structure would suggest<sup>6</sup>.

A study of griseofulvin (2) provides an example of the technique of "carrier-dilution", whereby the existence of an intermediate "X", suspected present in minute quantities, can be tested by feeding radioactive precursors and subsequently adding and reisolating synthetic "X". Hence the benzophenone (3), which did not acquire activity by this procedure, was shown to be an unlikely intermediate of griseofulvin<sup>7</sup>.

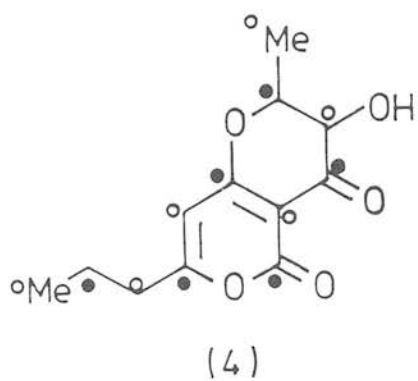
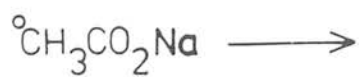
## 2.4 Use of N.M.R. Spectroscopy in Tracer Studies

This technique, of more recent and continuing rapid development, is of particular relevance to the work to be described later and will be discussed in some detail. Recent reviews<sup>8</sup> include many further examples of the application of n.m.r. spectroscopy to biosynthetic studies.

### 2.4.1 Use in Tracing Carbon

#### 2.4.1.1 $^{13}\text{C}$ N.m.r. Spectroscopy

Several comprehensive reviews<sup>9-11</sup> on the biosynthetic applications of  $^{13}\text{C}$  n.m.r. spectroscopy are available and the topic has been usefully summarised by Steyn<sup>12</sup>. Although some early work was carried out using the  $^{13}\text{C}$  satellite bands in the proton n.m.r. spectrum, this disadvantaged method has been superceded by direct observation of the  $^{13}\text{C}$  n.m.r. signal.  $^{13}\text{C}$  is the only n.m.r. active isotope of carbon (natural abundance = 1.1%, nuclear spin =  $\frac{1}{2}$ ) and under conditions of proton noise decoupling gives rise to a single line for each chemically non-equivalent carbon atom. Coincident lines are relatively rare due to the wide range of  $^{13}\text{C}$  chemical shifts observed for organic molecules. A prerequisite of biosynthetic studies is the assignment of each signal to the correct carbon position. This should obviously be independent of preconceived biosynthetic hypotheses. Spectral assignment may be far from trivial and, to this end, a whole battery of useful techniques has been devised<sup>11,12</sup>.





#### 2.4.1.2 Single $^{13}\text{C}$ -Labelling

The label from  $^{13}\text{C}$ -enriched precursors is traced by obtaining the proton noise decoupled (p.n.d.)  $^{13}\text{C}$  n.m.r. spectrum of the labelled metabolite and observing which signals show increased intensity relative to their intensity in the natural abundance spectrum. This direct method was first used<sup>13</sup> to study the incorporation of [1- $^{13}\text{C}$ ]- and [2- $^{13}\text{C}$ ]acetates into radicinin (4) and has since been widely adopted.

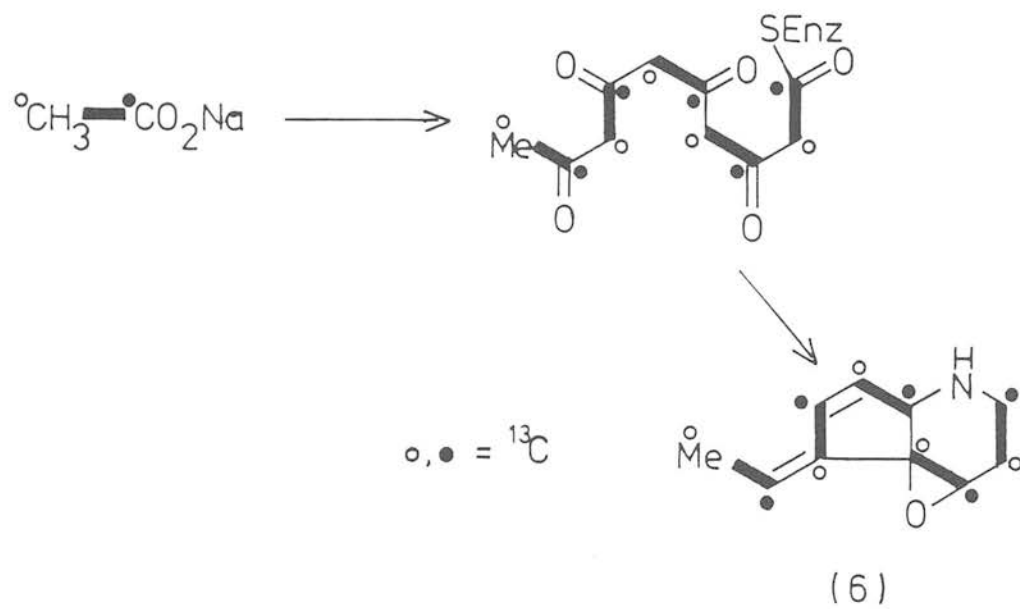
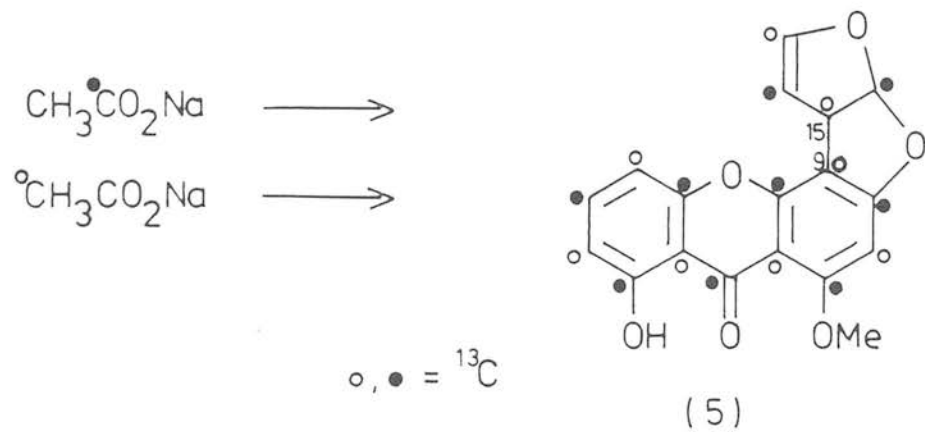
Quite apart from the experimental convenience of using a non-radioactive isotope, the main advantage of  $^{13}\text{C}$  n.m.r. studies over  $^{14}\text{C}$  radiolabelling is the absence of the need for chemical degradations to establish the sites of labelling. Suitable degradations are now anyway often superfluous in structure determination and hence unavailable, whereas  $^{13}\text{C}$  n.m.r. spectroscopy is routinely used for this purpose.

A disadvantage of  $^{13}\text{C}$  n.m.r. spectroscopy, one not shared by radiolabelling methods, is its insensitivity<sup>14</sup>. This is mostly due to the low energy of the transitions measured in n.m.r. generally, but the problem is more acute in the case of  $^{13}\text{C}$  than for protons because of the smaller magnetogyric ratio ( $\gamma$ ) of  $^{13}\text{C}$  (0.252 times that of protons). Sensitivity, atom for atom, is proportional to  $\gamma^3$ .

The natural abundance of  $^{13}\text{C}$  of 1.1% is a mixed blessing. It makes natural abundance spectra reasonably accessible without the complications of  $^{13}\text{C}$ - $^{13}\text{C}$  coupling; however, it also requires that the dilution (see below) of a 100%  $^{13}\text{C}$  single-labelled precursor be at most 200-fold. If a reasonably accurate assessment of enrichment is required, the dilution should be much less. For this reason, the method has found limited application in plant studies, where whatever is incorporated tends to become diluted by large amounts of endogenous material. Relatively large, *i.e.* non-tracer, quantities of labelled precursor are required even in studies using micro-organisms, and toxic effects, commonly manifested in reduced yields of metabolites, are sometimes observed. It is routine procedure to perform preliminary experiments with  $^{14}\text{C}$ -labelled precursors to check production and the dilution of label. This latter is given by<sup>11</sup>:

$$\begin{aligned} \text{Dilution per} &= \frac{\text{Specific Activity of Precursor}}{\text{Specific Activity of Metabolite}} \\ \text{labelled site} & \\ &\times \text{no. of labelled sites} \end{aligned}$$

$^{13}\text{C}$ - $^{13}\text{C}$  coupling, not observable in natural abundance spectra owing to the low probability of adjacent positions being labelled, may be observable in highly enriched material. For example, the detection of a  $^1J_{\text{CC}}$  coupling between C-9 and C-15 of sterigmatocystin (5) confirmed



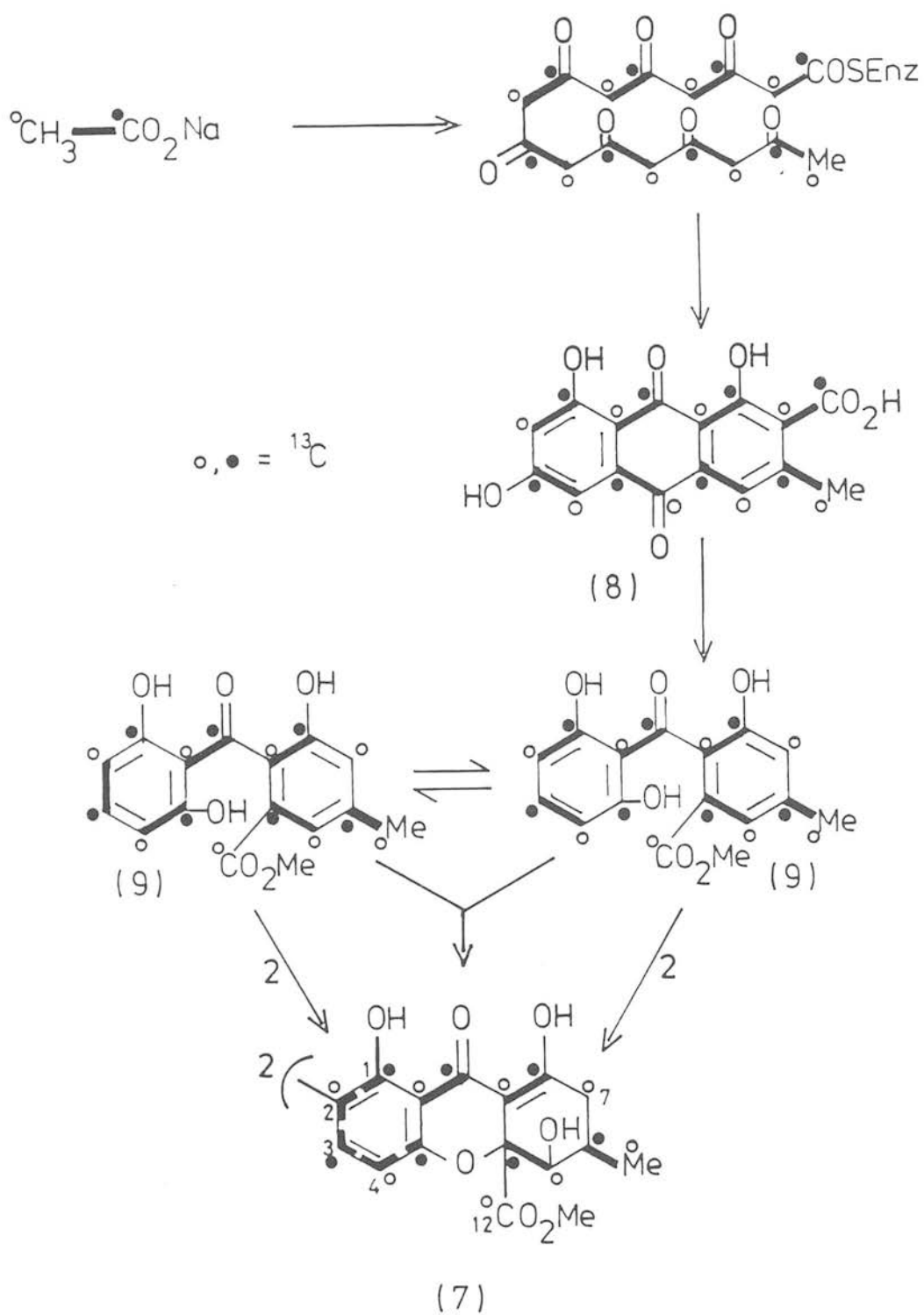
Scheme 2

these assignments and that both positions were labelled by [2- $^{13}\text{C}$ ]acetate<sup>15</sup>.

#### 2.4.1.3 Double $^{13}\text{C}$ -Labelling

A major extension to the  $^{13}\text{C}$  n.m.r. technique was introduced in 1973 by Seto *et al*<sup>16</sup> with the use of doubly  $^{13}\text{C}$ -labelled acetate in a study of dihydrolatumicidin (6) (Scheme 2). Using this precursor, adjacent carbon atoms derived from the incorporation of an intact acetate unit will exhibit  $^1\text{J}_{\text{CC}}$  coupling in the  $^{13}\text{C}$  n.m.r. spectrum of the enriched metabolite. Unless the dilution of acetate is low, no coupling between adjacent units will be observed. Thus all ten carbon positions of dihydrolatumicidin showed a  $^1\text{J}_{\text{CC}}$  coupling. On the basis of the sizes of the couplings the pairs derived from each intact unit could be identified, confirming a pentaketide labelling pattern as shown. The  $^1\text{J}_{\text{CC}}$  coupling is structure dependent, increasing with the "s" character of the atoms involved<sup>17</sup>.

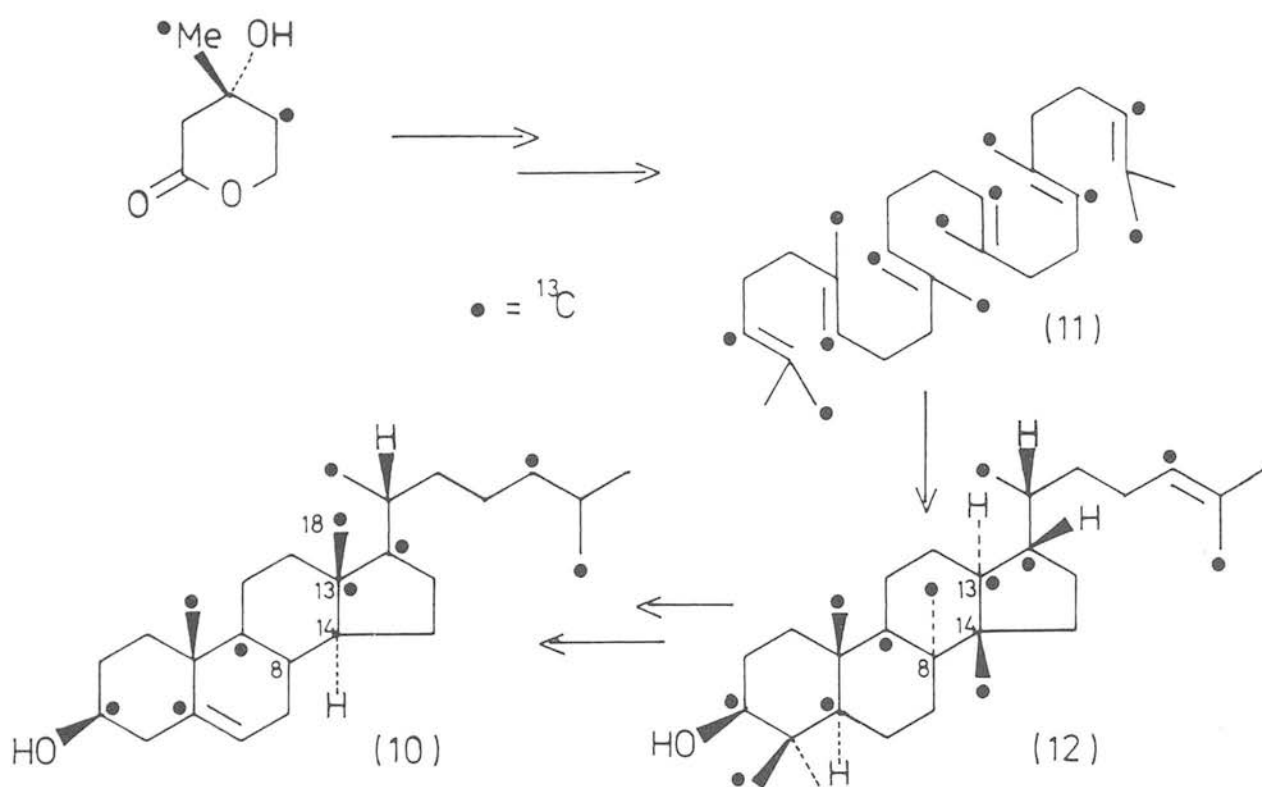
An alternative approach, first employed in the same study<sup>16</sup>, is the use of a 50:50 mixture of [1- $^{13}\text{C}$ ]- and [2- $^{13}\text{C}$ ]acetates, giving rise to  $^1\text{J}_{\text{CC}}$ ,  $^2\text{J}_{\text{CC}}$  and  $^3\text{J}_{\text{CC}}$  couplings between adjacent units. Very low dilutions are required and the method probably has more potential as a source of structural information rather than of biosynthetic insight. In contrast, the contiguous double labelling technique has found wide employment, partly because it extends the permissible dilution factor



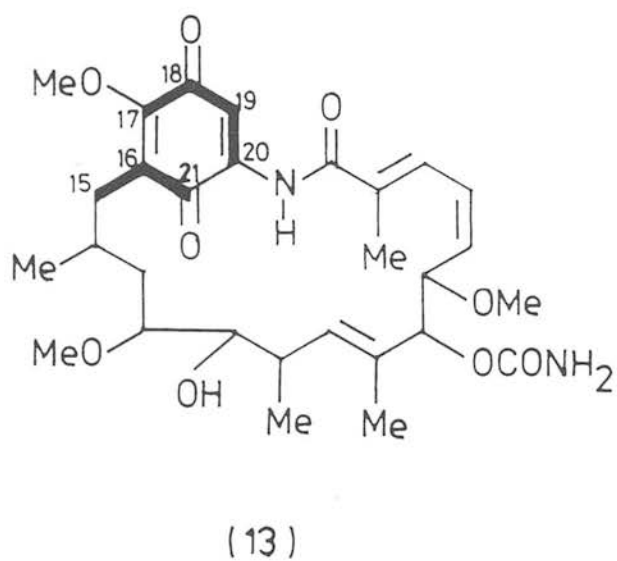
Scheme 3

to about 500<sup>9</sup>, but largely because of the extra information obtainable in respect of bond cleavages, rearrangements and symmetrical elements of intermediates. A study<sup>18</sup> of the polyketide, secalonic acid A (7), provides a good illustration. [1,2-<sup>13</sup>C<sub>2</sub>]Acetate labelled the molecule as shown (Scheme 3). C-7 and C-12, although labelled by [2-<sup>13</sup>C<sub>2</sub>]acetate, did not show coupling, indicating that the C-C bonds of their acetate units had been broken. C-2, C-3 and C-4 all showed two pairs of coupling satellites each, all of which were about half the intensity of those arising from other intact acetate units. These results were consistent with the proposed formation of secalonic acid A by ring-cleavage of an octaketide anthraquinone intermediate (8) to give a benzo-phenone species (9), the symmetrically substituted ring of which was free to rotate. The tetrahydroxanthone nucleus could then be formed with two different labelling patterns, indistinguishable without the extra <sup>1</sup>J<sub>CC</sub> coupling data.

Further examples of the diagnostic power of double <sup>13</sup>C-labelled precursors abound and examples are regularly reviewed<sup>8</sup>. The vast majority of studies with precursors multi-labelled contiguously with <sup>13</sup>C have employed [1,2-<sup>13</sup>C<sub>2</sub>]acetate. However, examples of the use of other such precursors such as [1,2,3-<sup>13</sup>C<sub>3</sub>]malonate<sup>19</sup>, [1,2-<sup>13</sup>C<sub>2</sub>]propionate<sup>20</sup>, [2,3-<sup>13</sup>C<sub>2</sub>]succinate<sup>21</sup>, [<sup>13</sup>C<sub>6</sub>]-glucose<sup>22</sup> and [4,5-<sup>13</sup>C<sub>2</sub>]mevalonate<sup>23</sup> have been published in recent years.



Scheme 4



Precursors containing two non-contiguous  $^{13}\text{C}$  labels which become adjacent during the course of biosynthesis have been used. An example is found in a study of cholesterol (10) using  $[4,6-^{13}\text{C}_2]\text{mevalonic acid}^{24}$  (Scheme 4). C-13 and C-18 both showed a  $^1\text{J}_{\text{CC}}$  coupling, (*ca.* 35 Hz) showing that they were derived from the same mevalonate unit of squalene (11) and confirming that the appearance of C-18 on C-13 occurs by a 1:2 methyl migration from C-14 of the initial cyclisation product (12) rather than by a 1:3 migration from C-8. As both labels are derived from the same precursor unit in an experiment of this nature, it is not necessary that the dilution of the precursor is exceptionally low.

#### 2.4.1.4 $^{13}\text{C}$ -Depleted Materials

Materials containing very low (0.1%) levels of  $^{13}\text{C}$  are available as a by-product of  $^{13}\text{C}$ -enrichment processes and it was suggested<sup>25</sup> that the incorporation of these could be detected by a reduction in the intensity of the "labelled" carbon resonances. Although it has since been pointed out<sup>26,9</sup> that this is impractical unless dilutions of less than two-fold can be achieved,  $^{13}\text{C}$ -depleted materials do have potential in biosynthetic studies. If the "background" abundance of  $^{13}\text{C}$  in the intermediates of a metabolic pathway can be reduced by using a  $^{13}\text{C}$ -depleted carbon source for the organism, then subsequent incorporation studies with  $^{13}\text{C}$ -enriched compounds will give meaningful results at precursor



dilutions which could not otherwise be tolerated. Thus, in a study<sup>22</sup> of the "m-C<sub>7</sub>N unit" of geldamycin<sup>na</sup> (13), low enrichment by [<sup>13</sup>C<sub>6</sub>]glucose relative to natural abundance <sup>13</sup>C was overcome by feeding a mixture of <sup>13</sup>C-enriched and <sup>13</sup>C-depleted glucoses to cultures of *Streptomyces hygroscopicus* growing in a medium already containing <sup>13</sup>C-depleted glucose. The n.m.r. spectrum of the isolated geldamycin indicated that C-15, C-16 and C-21 formed an intact C<sub>3</sub> unit (from phosphoenol pyruvate) and that C-17 to C-20 formed an intact C-4 unit (from erythrose-4-phosphate).

#### 2.4.1.5 Quantitative <sup>13</sup>C Measurements

In principle at least, quantitative values for the degree of enrichment by labelled precursors and intermediates can be determined by comparing the integrated intensities of different carbon resonances in the p.n.d. <sup>13</sup>C n.m.r. spectrum. The percentage of <sup>13</sup>C isotope above natural abundance has been defined<sup>27</sup> as:

%age enrichment =

$$\frac{1.1 \text{ (integrated intensity at labelled centre)}}{\text{(integrated intensity at unlabelled centre)}} - 1.1$$

and the specific incorporation as:

$$\text{Specific incorporation} = \frac{\% \text{ enrichment}}{\text{atom } \% \text{ } ^{13}\text{C in precursor}}$$

In practice, however, comparing signal heights or integrals is problematic and the complications have been widely discussed<sup>11,12,28</sup>. Due to the wide range of spin-lattice relaxation times ( $T_1$ 's) encountered in  $^{13}\text{C}$  n.m.r. spectroscopy and the variable effects of the nuclear Overhauser enhancement (n.O.e.) induced by proton-decoupling, the line intensities in a  $^{13}\text{C}$  spectrum usually show wide variation even in a natural abundance spectrum. Fourier transform n.m.r. spectrometers exacerbate this problem using normal pulse sequences and add another one: the digitization of data often results in a poorly defined peak shape. The first problem can be circumvented to an extent by comparing unenriched and enriched resonances of carbons in a similar environment<sup>27</sup>, but a better solution is to obtain natural abundance and enriched spectra under identical conditions, normalize both spectra to a standard which is known to be unlabelled, and compare the intensities of the lines in the two spectra directly<sup>11</sup>. An alternative approach is to add a paramagnetic relaxation agent such as chromium tris-acetoacetate which suppresses the n.O.e. and shortens the  $T_1$ 's. It may also be necessary to use "inverse gated" proton-decoupling in which the n.O.e. is reduced by only decoupling during acquisition. Further alleviation of the effects of long  $T_1$ 's may be achieved by the use of a long delay between pulses.

The second problem, that of data handling, manifests itself in random variations in intensities in the lines of spectra obtained from the same sample under the same experimental parameters. These variations can be reduced by using more data points to define the spectrum or by acquiring the spectrum several times and averaging the results. As can be seen, the price to be paid in instrument time, in return for reliable enrichment data, is considerable and some compromise is generally required.

A formula for calculating the enrichment from the relative intensities of the centre and satellite lines of a carbon position labelled by  $[1,2-^{13}\text{C}_2]\text{acetate}$  has been proposed<sup>29</sup>, applicable when the chance of any single molecule of metabolite containing adjacent labelled acetate units is small. The method has, however, been criticised<sup>30</sup> on the grounds that spin-lattice relaxation by bonded  $^{13}\text{C}$  makes an important contribution to the overall relaxation rate of non-protonated carbon atoms. In addition intensity errors arise in the case where a closely coupled pair of  $^{13}\text{C}$  atoms form an AB type system.

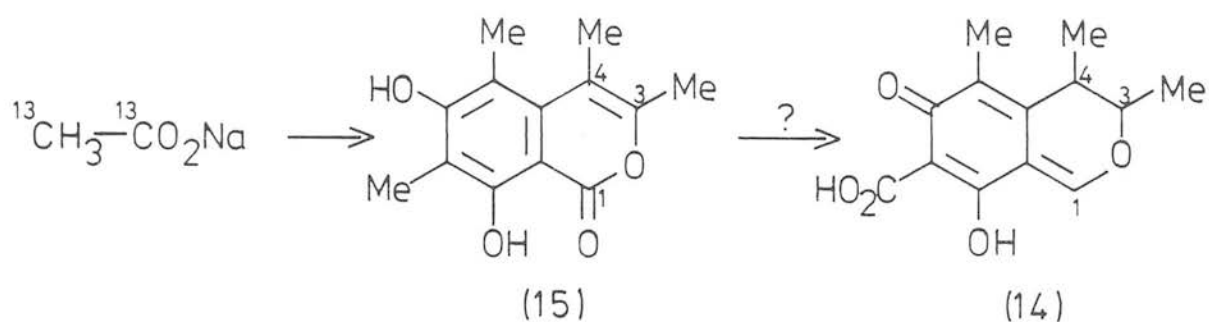
#### 2.4.2 Use in Tracing Hydrogen

##### 2.4.2.1 Hydrogen Isotopes

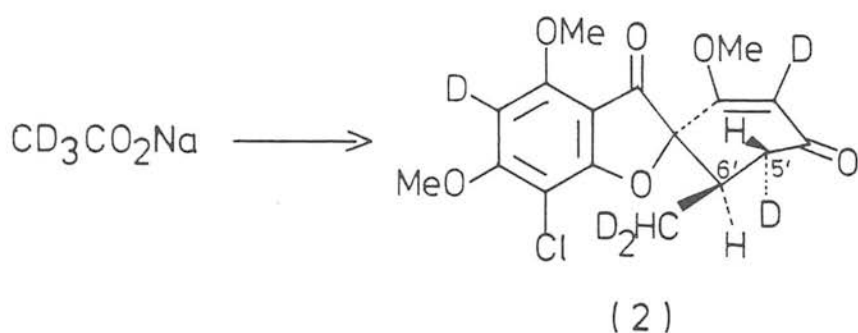
The use of n.m.r. spectroscopy to trace hydrogen in biosynthetic pathways has been reviewed<sup>31</sup> and a further discussion<sup>32</sup> also covers this subject in some detail.

Table 1      Some properties of hydrogen isotopes relevant  
to their use as n.m.r. tracers

	Protium	Deuterium	Tritium
Mass	1.008	2.014	3.016
Natural abundance	99.984%	0.016%	1 in $10^{17}$
Nuclear spin	$\frac{1}{2}$	1	$\frac{1}{2}$
Relative gyromagnetic ratio	1.000	0.154	1.067
Relative sensitivity	1.000	0.00365	1.21
Resonant frequency (23 kG)	100 MHz	15.4 MHz	106.7 MHz



Scheme 5



All three of the isotopes of hydrogen are n.m.r. active and each will be briefly considered in turn. Some of the salient properties of each nucleus are given in Table 1. Because of the relatively large change in fractional mass occurring at the site of labelling when using hydrogen isotopes, the results may need cautious interpretation although the kinetic isotope effect may sometimes be put to good use. For this reason deuterium is considered a better "tracer" for hydrogen than is tritium since the properties of deuterium-labelled compounds are closer to those of the corresponding protium compounds.

#### 2.4.2.2 Protium

Although, in normal circumstances, protium, due to its ubiquity, cannot be used as a tracer, a study of citrinin (14) biosynthesis where *Penicillium citrinum* was grown on a medium based on D<sub>2</sub>O is a notable exception<sup>33,34</sup>.  $^{13}\text{CH}_3$   $^{13}\text{CO}_2\text{Na}$  was fed to the culture and the presence of acetate-derived hydrogen was inferred from the appearance of the  $^{13}\text{C}$  n.m.r. spectrum. Carbon atoms in the metabolite derived directly from the acetate were distinguished from those originally within the glucose carbon source by the  $^1\text{J}_{\text{CC}}$  couplings exhibited by the former. Thus the isocoumarin (15), known to be specifically incorporated into citrinin, was shown, by the presence of acetate-derived protium at C-4, to be a non-obligatory intermediate (Scheme 5).

### 2.4.2.3 Deuterium

#### (i) Direct Method

Due to its low natural abundance and relative cheapness, deuterium ( $^2\text{H}$ ) has been the most widely used of the hydrogen isotopes for biosynthetic work. Because it is a quadrupole nucleus and thus very efficiently relaxed, the spectral lines are rather broad and this, combined with the small chemical shift range for hydrogen nuclei, often results in poorly resolved spectra. However, the rapid relaxation and lack of any n.O.e. effect mean that accurate integration of  $^2\text{H}$  n.m.r. spectra is possible. Much greater dilutions are tolerable than in the case of  $^{13}\text{C}$ -labelling: a 100%  $^2\text{H}$ -labelled precursor may be diluted some 6000-fold and still result in a doubling of intensity over the corresponding natural abundance signal.

One of the earliest biosynthetic applications of  $^2\text{H}$  n.m.r. spectroscopy was in a study of griseofulvin (2)<sup>35</sup>. The  $5'\alpha$  and  $5'\beta$  hydrogens could be resolved in the  $^2\text{H}$  n.m.r. spectrum and it was shown that  $^2\text{H}$  from [ $^2\text{H}_3$ ]acetate was present only in the  $5'\alpha$  position. This potential ability to distinguish between diastereotopic hydrogens is one of the most useful features of  $^2\text{H}$  n.m.r. spectroscopy. The information obtained by this method, however, represents the sample as a whole; hence, the conclusion that the 6'-methyl group was labelled as  $-\text{CHD}_2$  is not inescapable: the observations could also have been

explained by an equal mixture of molecules labelled  $-CHD_2$ ,  $-CH_2D$  and  $-CD_3$ .

(ii) Indirect Methods

A recent innovation is the use of  $^{13}C$  as a "reporter nucleus" in deuterium-labelling studies<sup>36</sup>. The presence of deuterium  $\alpha$  or  $\beta$  to a  $^{13}C$  atom can be deduced from the appearance in the p.n.d.  $^{13}C$  n.m.r. spectrum of  $^{13}C-^2H$  coupling and/or an isotopically induced shift. It should be noted that the previously described limitations of  $^{13}C$  n.m.r. spectroscopy, *i.e.* insensitivity and quantitative inaccuracy, apply also to these experiments. However the wide spectral range of  $^{13}C$  is a distinct advantage.

As the phenomenon of isotopically induced shifts also makes feasible the indirect detection of oxygen label (see below) by n.m.r. spectroscopy, some general notes on the effect, arising out of a comprehensive discussion<sup>37</sup>, are included here.

- (a) Substitution by a heavy isotope shortens the average length of the bond holding the isotope, and, less so, the remaining bonds of the molecule. This almost always shifts the n.m.r. signal of neighbouring nuclei to high field although the magnitude of the shift is inversely dependent on the remoteness of the substitution.
- (b) The shift is largest where the fractional change in mass is largest and is also roughly proportional to the number of atoms substituted.

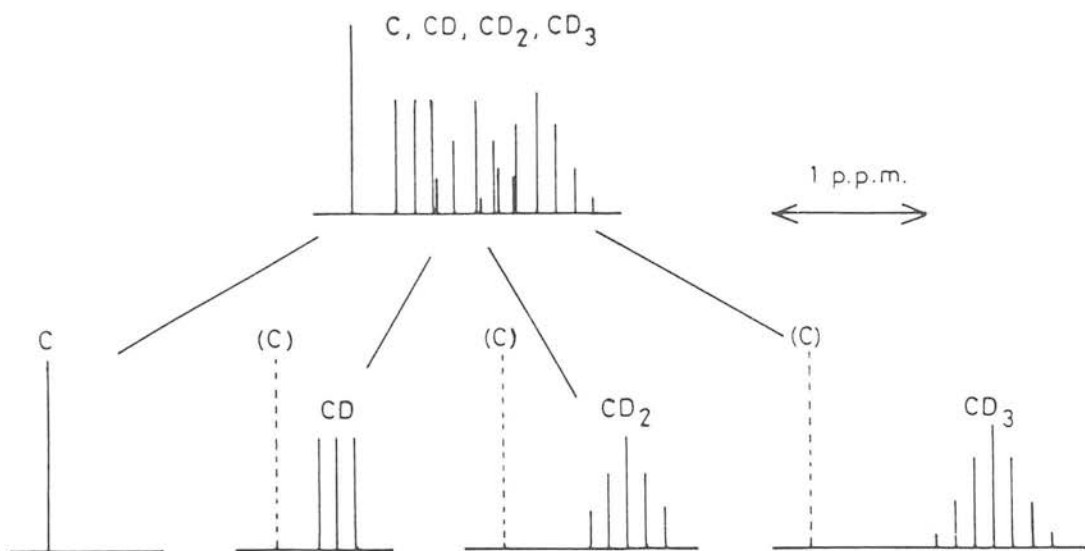


Figure 1 Expected appearance of the  $^1\text{H}$ -decoupled  $^{13}\text{C}$  n.m.r. resonances of carbons bearing zero, one, two or three deuterons (alpha-shifted)

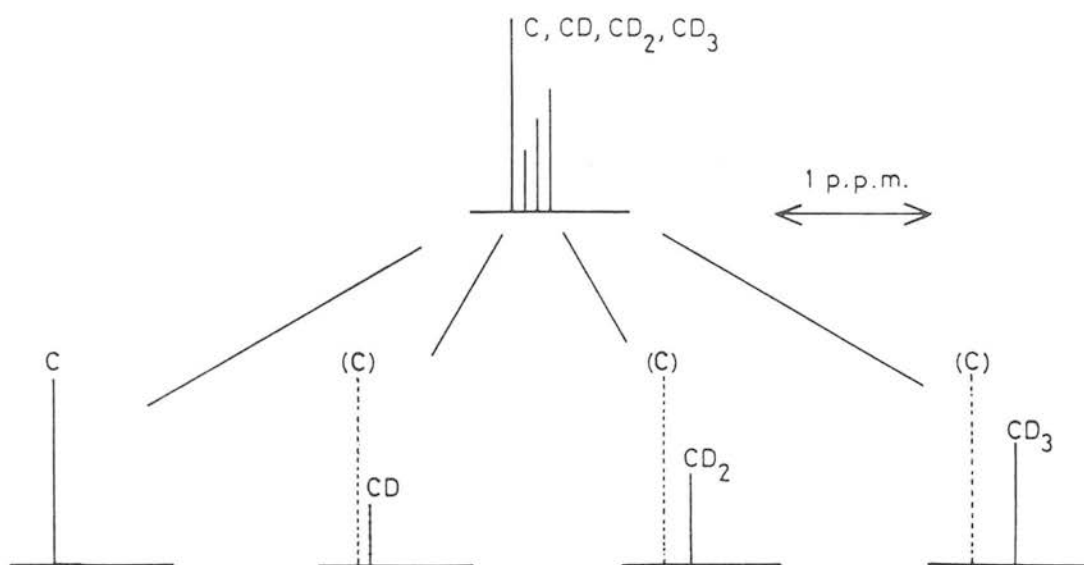


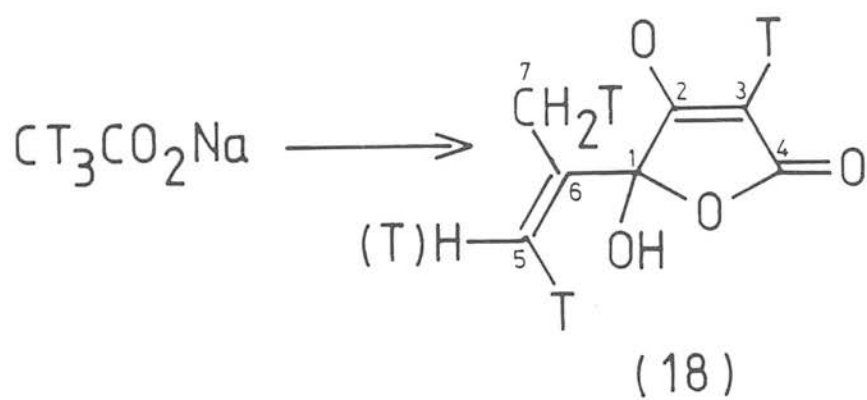
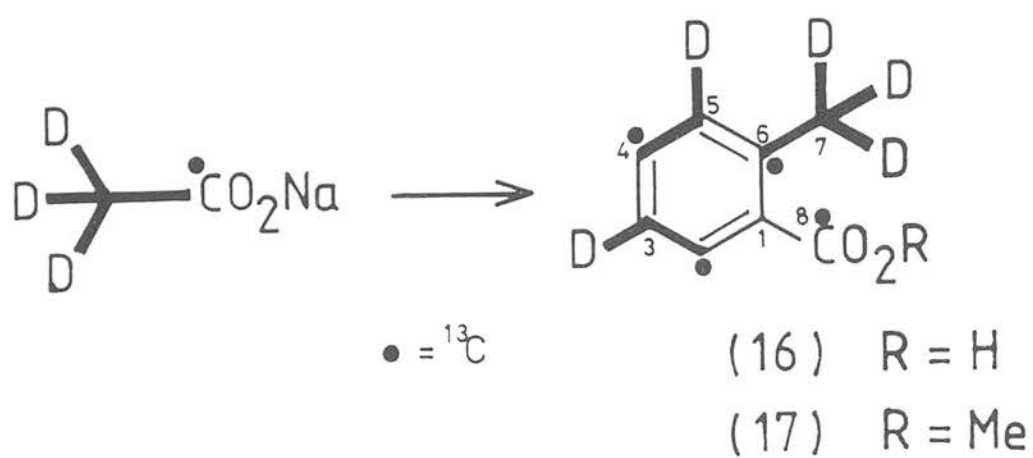
Figure 2 Expected appearance of the  $^1\text{H}$ -decoupled  $^{13}\text{C}$  n.m.r. resonances of carbons two bonds removed from zero, one, two or three deuterons (beta-shifted)



(c) The magnitude of the shift decreases with an increase in "s" character of the bond holding the isotope and depends also on the resonant nucleus, correlating with the range of chemical shifts observed for that nucleus.

(iii) The Alpha-Shift Technique<sup>31</sup>

In this technique, the deuterium label is directly attached to the  $^{13}\text{C}$  nucleus in the precursor molecule. The p.n.d.  $^{13}\text{C}$  n.m.r. spectrum of the enriched metabolite shows, for carbon atoms which have retained deuterium label, a series of resonances upfield of the normal singlet. The presence of each deuterium shifts the centre of the carbon resonance by 0.3 - 0.6 ppm to low frequency and spin-spin coupling ( $^1J_{\text{CD}}$ ) gives rise to a characteristic multiplet pattern; hence CD appears as a triplet centred 0.3 - 0.6 ppm upfield of the normal singlet,  $\text{CD}_2$  gives a quintet centred 0.6 - 1.2 ppm upfield and  $\text{CD}_3$  gives a septet (Figure 1). Shifted signals arising from carbons which bear no protium suffer reduced signal to noise ratio caused by poor relaxation and lack of n.O.e. enhancement, a disadvantage of the method which is compounded by the multiplicities due to coupling. Deuterium decoupling can assist in this last respect. However, information not obtainable by direct  $^2\text{H}$  n.m.r. spectroscopy, such as the distribution of label as  $\text{CH}_2\text{D}$ ,  $\text{CHD}_2$  and  $\text{CD}_3$  (see above) and the integrity of carbon-hydrogen bonds during biosynthesis, may be gained.



(iv) The Beta-Shift Technique

Many of the problems associated with directly attached deuterium are avoided by placing the deuterium label two bonds away from the  $^{13}\text{C}$  reporter nucleus. The isotope shift, although reduced, is still observable, and as  $\beta$ -hydrogens only contribute markedly to the relaxation of non-protonated  $^{13}\text{C}$  nuclei, the shifted signals otherwise retain any n.O.e. effect also experienced by the unshifted signals on proton-decoupling. As geminal carbon-proton coupling constants are generally small anyway, and carbon-deuteron couplings are over six times smaller again, the shifted signals are effectively singlets (Figure 2), even without deuterium decoupling, and this gives a further increase in the signal to noise ratio compared with the corresponding  $\alpha$ -shift experiment.

The method was first applied to biosynthesis in a study of 6-methylsalicylic acid (16)<sup>38</sup>. [ $^2\text{H}_3$ , 1- $^{13}\text{C}$ ]-acetate was fed to cultures of *Penicillium griseofulvum*. The p.n.d.  $^{13}\text{C}$  n.m.r. spectrum of the methyl ester (17) of the resulting 6-MSA showed shifted signals for C-2, C-4 and C-6, corresponding to the presence of deuterium label at positions 3, 5 and 7 respectively. Thus the integrity of an acetate unit (heavy lines) can be established in certain cases without recourse to a double  $^{13}\text{C}$ -labelled experiment.

Unlike the  $\alpha$ -shifts, the  $\beta$ -shifts show a marked dependence on both the stereochemistry<sup>39</sup> of the  $^2\text{H}$  label and the functionality of the  $^{13}\text{C}$  reporter nucleus. Carbonyl resonances, for instance, may show irresolvable<sup>40</sup> or even downfield<sup>39</sup> shifts and the three upfield resonances establishing a "starter unit" methyl may also appear as one broad signal<sup>38,40</sup>.  $\beta$ -shifts do, however, appear to be additive<sup>39</sup>.

#### 2.4.2.4 Tritium

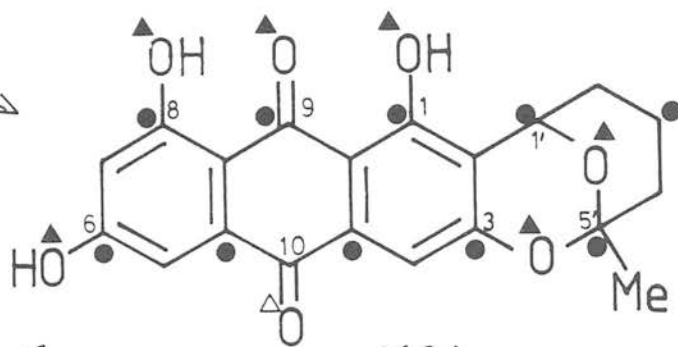
Although best known as a radioactive tracer, the tritium nucleus has ideal magnetic characteristics for use as an n.m.r. label<sup>31</sup>. As the natural abundance is practically zero and the magnetogyric ratio is the highest yet found for any nuclide, tritium n.m.r. spectroscopy is uniquely sensitive, by the standards of other n.m.r. methods of tracing isotopes. The chemical shift values and coupling constants are very close to those of the corresponding proton spectrum so assignments can first be made on this basis. Accurate integration is also possible<sup>41</sup>. The utility of the method in biosynthetic investigation was first demonstrated in a study<sup>42</sup> of penicillic acid (18), a metabolite of *Penicillium cyclopium*. Sodium [ $^3\text{H}$ ]acetate of high specific activity was incorporated and the  $^3\text{H}$  n.m.r. spectrum of the enriched metabolite (18) showed that the indicated positions carried tritium. The signal due to the allylic methyl group appeared as a triplet,  $J_{\text{HT}} = 15.5$  Hz, owing to coupling of the single tritium with the two protons. An

interesting observation was the stereospecific retention of tritium at the more upfield (*trans* to methyl) of the vinylic methylene hydrogens. The high resolution of tritium n.m.r. spectroscopy was a distinct advantage in this study. Although samples containing only 1 mCi of activity can give rise to satisfactory spectra, it may be necessary, especially using a simple precursor like acetate, to use relatively large amounts of tritium label: in the above study<sup>42</sup> nearly 0.5 Ci were employed. Possibly for this reason, tritium n.m.r. is still a rarely used technique.

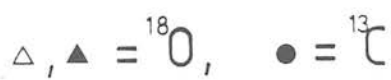
#### 2.4.3 Use in Tracing Oxygen

##### 2.4.3.1 <sup>17</sup>O Labelling

Of the isotopes of oxygen, only one, <sup>17</sup>O (natural abundance 0.037%), possesses nuclear spin (5/2) and thus gives rise to an n.m.r. spectrum. Unfortunately, it is an insensitive nucleus which gives broad lines due to quadrupolar relaxation. As it is also expensive, its use in biosynthetic studies has been very limited. The incorporation of acetate-derived oxygen into citrinin<sup>43</sup> (14) was followed by <sup>17</sup>O n.m.r. spectroscopy and the technique was also used to demonstrate that no oxygen was lost from the L- $\alpha$ -aminoadipyl section of the tripeptide precursor of isopenicillin N in the biosynthesis of the latter<sup>44</sup>.



(19)



#### 2.4.3.2 $^{18}\text{O}$ Labelling

Until recently then, the biosynthetic origin or fate of oxygen was determined almost exclusively by mass-spectrometry, and locating the position of a label, present perhaps in only low concentration, was difficult. It was known that the heaviest oxygen isotope,  $^{18}\text{O}$ , (natural abundance - 0.204%) was capable of inducing resolvable isotope shifts in the n.m.r. spectra of certain other elements and this had been exploited in biological studies, *e.g.* the mechanism and kinetics of enzymatic phosphoryl group transfer had been investigated *via* the  $^{18}\text{O}$  isotope shift in the  $^{31}\text{P}$  n.m.r. spectrum<sup>45</sup>. The detection<sup>46</sup> of such an isotope shift in the  $^{13}\text{C}$  spectrum provided a more general technique for biosynthetic research, first employed in a study<sup>47</sup> of averufin (19), implicated as an intermediate in aflatoxin biosynthesis. *Aspergillus parasiticus*, when grown under an atmosphere containing  $^{18}\text{O}_2$  gas, produced averufin, the  $^{13}\text{C}$  n.m.r. spectrum of which showed an isotopically shifted resonance for C-10 only. In an experiment feeding with  $[1-^{13}\text{C}, ^{18}\text{O}_2]\text{acetate}$ , carbons 1, 3, 6, 8, 9 and 1' all showed prominent shifted signals. C-5' showed only a very small shifted signal<sup>48</sup> due to the small percentage of molecules which carried  $^{18}\text{O}_2$  at either of the ketal positions and also, fortuitously, were labelled with  $^{13}\text{C}$  at the 5' position. Thus, at the incorporation levels typically achieved with early precursors, carbon-oxygen bonds which have been

preserved intact throughout the course of biosynthesis can be distinguished from those which have arisen between precursor units.

Application of the  $^{18}\text{O}$ -shift technique has been rapid and the method and its applications have been the subject of a review<sup>48</sup>. The shifts show a marked dependence on structure<sup>49,50</sup>, which could be of use in  $^{13}\text{C}$  spectral assignments, but they are not generally much larger than 0.05 ppm.



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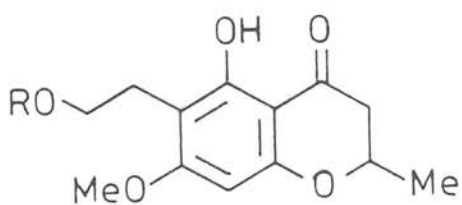
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Chapter 3

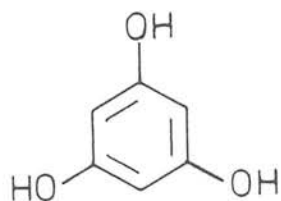
Structural Revision, Synthesis and

Biosynthesis of LL-D253 $\alpha$

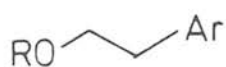


(1)  $R = H$

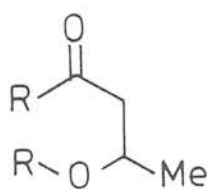
(9)  $R = COCH_3$



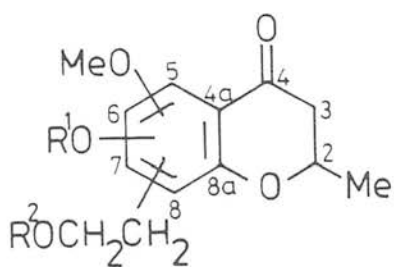
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(3)



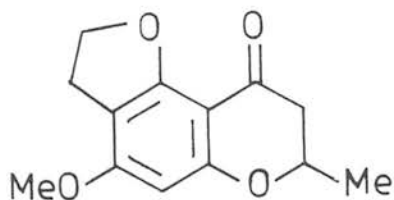
(4)



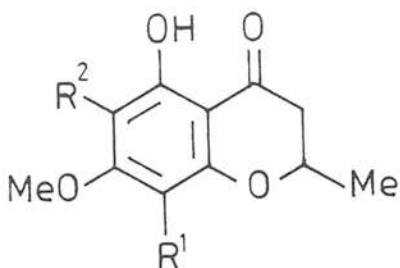
(5)  $R^1 = Me, R^2 = H$

(6)  $R^1 = R^2 = H$

(7)  $R^1 = R^2 = COCH_3$



(8)



(1)  $R^1 = H, R^2 = CH_2CH_2OH$

(10)  $R^1 = CH_2CH_2OH, R^2 = H$

### 3. Structural Revision, Synthesis and Biosynthesis of LL-D253 $\alpha$

#### 3.1 Background Information: Structure

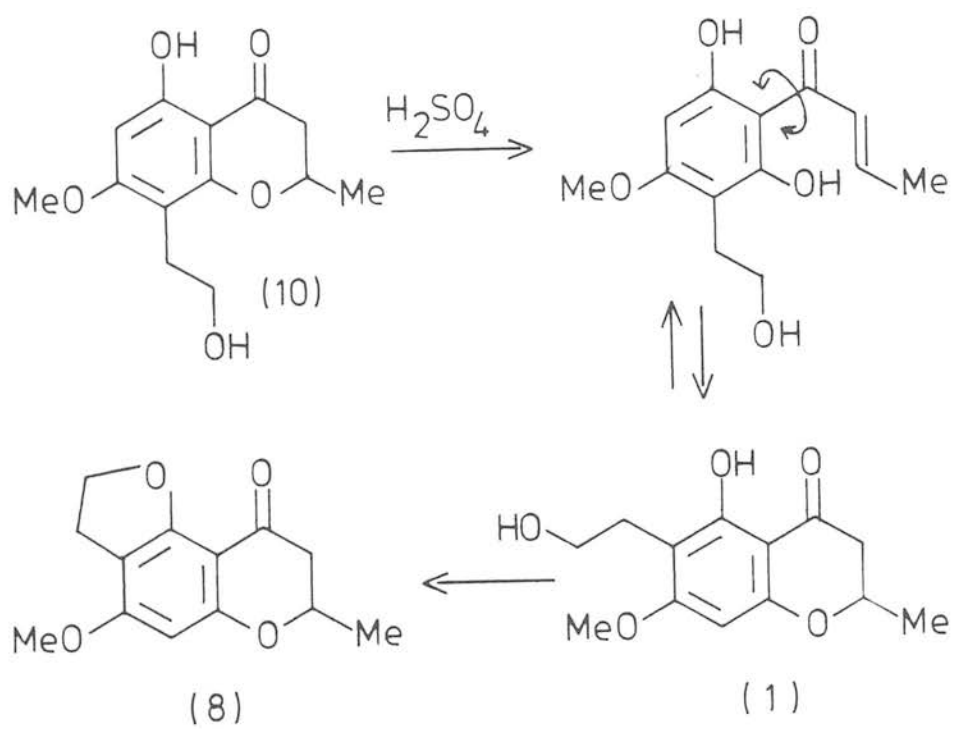
The isolation of a 4-chromanone metabolite, LL-D253 $\alpha$ , from *Phoma pigmentivora*, was first reported in 1972 and a structure (1) was proposed. This structure was based on spectroscopic evidence and on the results of chemical degradations. Fusion of LL-D253 $\alpha$  with sodium hydroxide yielded phloroglucinol (2), thus establishing the presence in the metabolite of a 1,3,5-trioxygenated benzene ring. Two further fragments, (3) and (4), were identified from the proton n.m.r. spectrum of the monomethyl ether (5); this was also shown to possess two aromatic methoxyls. Given the partial structure (6), the problem was reduced to one of placement of the three substituents on the aromatic ring. Two observations indicated to the authors that the phenolic hydroxyl occupied the position *peri* to the carbonyl, *i.e.* on C-5. Firstly, the carbonyl infra-red band, originally at the fairly low frequency of 1655 cm<sup>-1</sup> in LL-D253 $\alpha$ , appeared at 1699 cm<sup>-1</sup> in the diacetate (7). Secondly, an ultra-violet maximum, at 287 nm in methanol, shifted to 325 nm in basic solution and became much stronger.

If the phenolic hydroxyl was at C-5, the methoxyl group had to be at C-7 to give 1,3,5 oxygenation. As treatment of LL-D253 $\alpha$  with sulphuric acid yielded a dihydrofuran, formulated as structure (8), the ethanolic side-chain had to be at C-6. In addition, LL-D253 $\alpha$  gave a positive Gibbs test. This is generally taken to indicate that the ring-position *para* to a phenolic hydroxyl bears only hydrogen<sup>2</sup>. Thus, the American group<sup>1</sup> arrived at structure (1) for LL-D253 $\alpha$ .

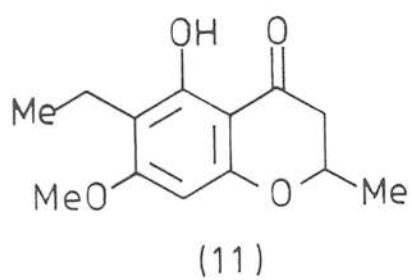
The dihydrofuran (8) was also identified as a naturally occurring compound (LL-D253 $\gamma$ ) in the filtrate of agitated fermentations of *P. pigmentivora*. Still fermentations gave the aliphatic monoacetate, LL-D253 $\beta$  (9), in addition to the parent compound (1).

LL-D253 $\alpha$ , as it later transpired, was also isolated from a *Phoma* strain by a Japanese group<sup>3</sup>. Ultra-violet and infra-red data again led to the assumption of an *ortho*-hydroxyarylketone structure (1 or 10) although in this case the carbonyl band, quoted at 1640 cm<sup>-1</sup>, was compared with that of the dimethyl ether at 1685 cm<sup>-1</sup>. Apparently on the basis of nuclear Overhauser effects, the structure (10) with the ethanolic side-chain at C-8 was initially favoured. After publication of the American results<sup>1</sup>, however, the American and Japanese compounds were shown to be identical.





Scheme 1



Before revising the structure of their sample of LL-D253 $\alpha$  to (1), the Japanese group considered the possibility that a Wessely-Moser rearrangement<sup>5</sup> could result in the formation of the dihydrofuran (8) from the C-8 substituted 4-chromanone (10) (Scheme 1). Such acid-catalysed rearrangements of 4-chromanones were alleged<sup>3</sup> to be well-known although no reference was given and this appears to be true only in the rather special case of flavanones<sup>6,7</sup>.

However, as the dihydrofuran produced by sulphuric acid treatment of LL-D253 $\alpha$  retained the optical activity observed in the naturally occurring metabolite, LL-D253 $\gamma$ , the rearrangement possibility was discounted and structure (1) was considered established for LL-D253 $\alpha$ . Both groups determined the absolute configuration of LL-D253 $\alpha$  at C-2 as *R* by circular dichroism.

LL-D253 $\alpha$  has also been isolated from *Sclerotinia fructigena* and *Phoma violacea*; the latter species also produced LL-D253 $\gamma$  and another closely related compound which possesses an ethyl group instead of an ethanolic group and was given structure (11)<sup>8</sup>.

Table 1  $^{13}\text{C}$  n.m.r. data for LL-D253 $\alpha$  and its diacetate

$\delta$ (p.p.m.)		Multiplicity <sup>c</sup>	Assignment <sup>d</sup>
(LL-D253 $\alpha$ ) <sup>a</sup>	(diacetate) <sup>b</sup>		
20.5	20.0	q	2-methyl
	20.2	q	acetate
	20.3	q	methyls
26.5	22.7	t	C-1'
45.2	44.9	t	C-3
55.4	55.5	q	O-methyl
60.1	62.2	t	C-2'
73.3	73.6	d	C-2
92.7	98.5	d	C-8
104.5	108.8	s	C-4a, 6
104.7	110.2	s	
159.9	154.5	s	
162.0	159.3	s	C-5, 7, 8a
162.2	161.5	s	acetate carbonyls
	168.0	s	
	170.2	s	
188.5	189.4	s	C-4

<sup>a</sup>Solution in  $\text{d}_6$  DMSO

<sup>b</sup>Solution in  $\text{CDCl}_3$

<sup>c</sup>From s.f.o.r.d. spectra

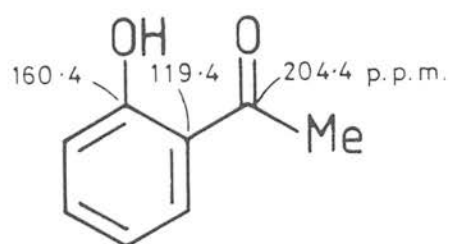
<sup>d</sup>Based on original structure (1), chemical shifts and proton multiplicities only.

### 3.2 Structural Revision and $^{13}\text{C}$ Spectral Assignment of LL-D253 $\alpha$

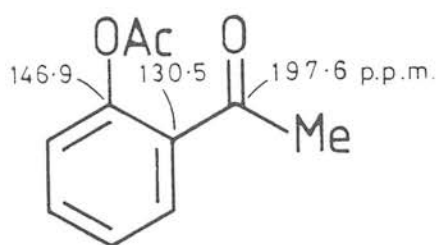
The structure (1) put forward<sup>1,3</sup> for LL-D253 $\alpha$  was entirely consistent with the data available and was taken to be correct when biosynthetic studies of LL-D253 $\alpha$  were first undertaken (Section 3.4). These studies first necessitated an unambiguous assignment of the  $^{13}\text{C}$  n.m.r. spectrum.

LL-D253 $\alpha$  itself, however, proved to be inconvenient for tracer studies using  $^{13}\text{C}$  n.m.r. spectroscopy. Firstly, it was virtually insoluble in the preferred n.m.r. solvents. Secondly, the  $^{13}\text{C}$  n.m.r. spectrum (Table 1 ) included two pairs of nearly coincident resonances. Thirdly, it turned out that the  $^1\text{J}_{\text{CC}}$  couplings of LL-D253 $\alpha$  enriched from [1,2- $^{13}\text{C}_2$ ]acetate could not be unambiguously matched up. The  $^{13}\text{C}$  n.m.r. assignments were therefore carried out on the diacetate which was easily prepared by heating LL-D253 $\alpha$  with acetic anhydride and pyridine.

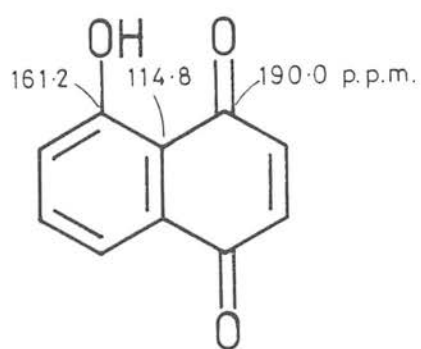
Chemical shift data<sup>9,10</sup>, and the multiplicities observed on single-frequency, off-resonance proton-decoupling, allowed the assignment of the diacetate  $^{13}\text{C}$  resonances below 100 p.p.m. (Table 1 ). In addition, the C-4 carbonyl resonance was easily identified as the lowest field signal, and the two signals around 170 p.p.m. were assigned to the acetate carbonyls. However, the remaining five signals, due to the non-protonated aromatic



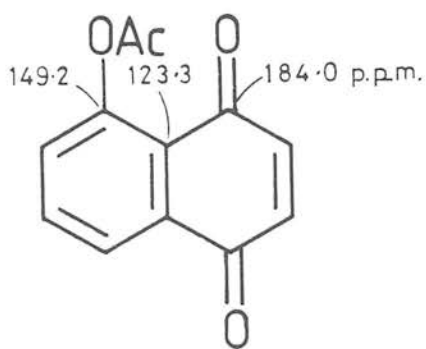
(12)



(13)



(14)



(15)

carbons, could not be individually identified without further information although they could be divided into two groups, oxygenated and non-oxygenated, on the basis of chemical shifts. These assignments were crucial to the biosynthetic studies.

It was hoped that comparison of the aromatic carbon signals of LL-D253 $\alpha$  and of the diacetate would assist in this respect. Large changes in the  $^{13}\text{C}$  chemical shift values of systems such as (1) have been observed when the chelation of the carbonyl group by the *peri*-hydroxylic function is removed. The chemical shifts of the acetophenone (12) and its acetate derivative (13) were used to help assign the  $^{13}\text{C}$  spectra of juglone (14) and its acetate (15)<sup>11</sup>. By analogy with these observations, it was expected that, on acetylation, the carbonyl resonance of LL-D253 $\alpha$  should move about 7 p.p.m. upfield, the C-4a resonance should move about 11 p.p.m. downfield, and the C-5 resonance should move about 13 p.p.m. upfield. In fact, only minor changes were observed (Table 1 ) and these were not helpful in determining the assignments of the aromatic carbons. Some disagreement between the anticipated and observed chemical shift changes might have been expected as a result of the change in n.m.r. solvent but the extent of the discrepancy was nevertheless striking. On the other hand, the carbonyl infra-red absorption did move from 1652  $\text{cm}^{-1}$  to 1689  $\text{cm}^{-1}$  as would

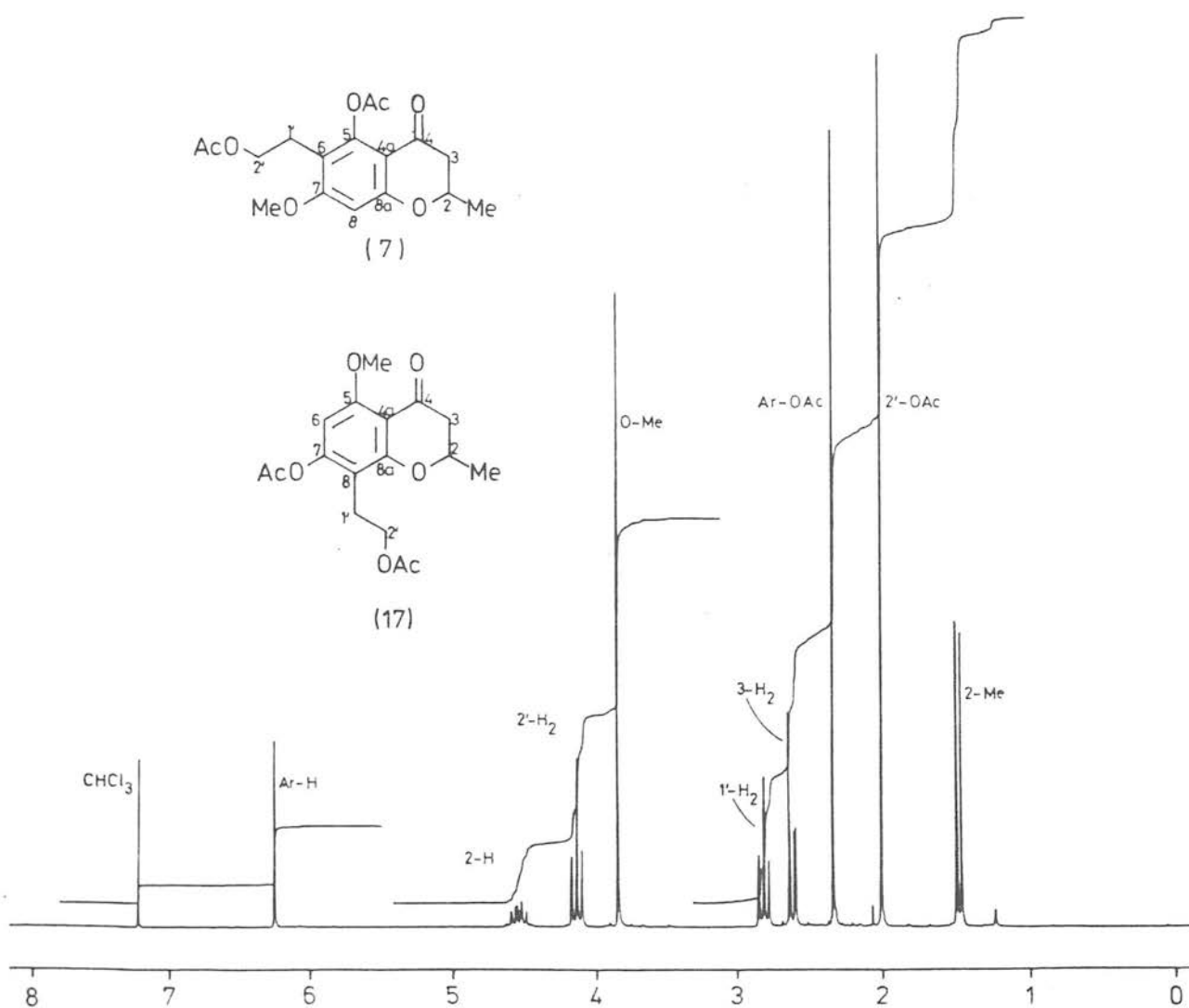
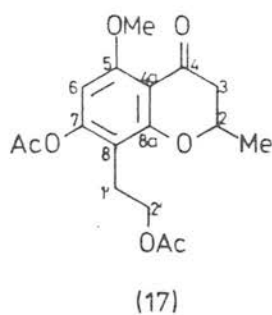
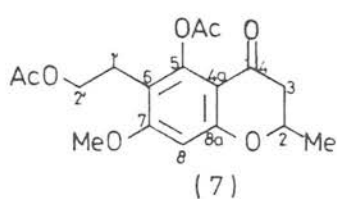
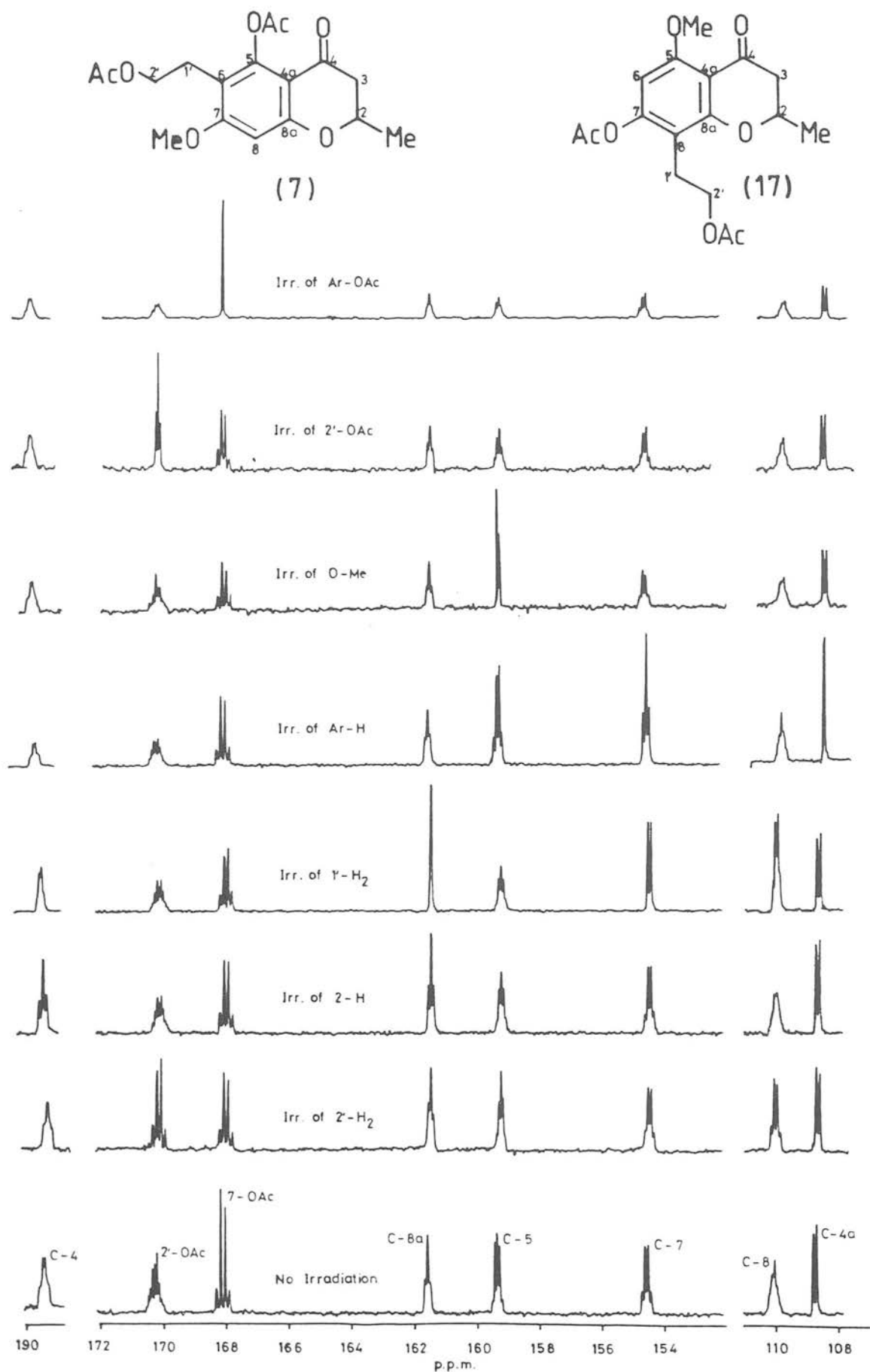


Figure 1 The 200 MHz  $^1\text{H}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate (in  $\text{CDCl}_3$ )



**Figure 2** The low-field region of the fully <sup>1</sup>H-coupled 50 MHz <sup>13</sup>C n.m.r. spectrum of LL-D253α diacetate and the results of selective <sup>1</sup>H-decoupling experiments (in CDCl<sub>3</sub>).



be expected on loss of chelation and as reported by the American group<sup>1</sup>.

Although acetylation did not produce all the expected changes in the  $^{13}\text{C}$  spectrum, the unassigned aromatic signals of the diacetate were better separated than were the corresponding signals of LL-D253 $\alpha$  itself. In addition, the proton n.m.r. spectrum of the diacetate (Figure 1) was easily interpreted and was devoid of overlapping signals; two useful features for selective proton-decoupling.

The effects of a series of such decoupling experiments on the low-field region of the fully proton-coupled  $^{13}\text{C}$  n.m.r. spectrum are shown in Figure 2. It was soon apparent that the original structure (7) could not be reconciled with these results. In particular, the decoupling of the methine proton, H-2, markedly sharpens the triplet at 161.5 p.p.m. which must thus be due to C-8a. (The only other signal affected is that of the C-4 carbonyl, at 189.4 p.p.m.) However, decoupling of the benzylic methylene hydrogens collapses this same signal, at 161.5 p.p.m., to a broad singlet. The hydroxyethyl side-chain must therefore occupy the C-8 position of the aromatic nucleus. Decoupling the benzylic hydrogens also affected two other aromatic signals: that at 154.5 p.p.m. (quartet to doublet) and that at 110.2 p.p.m. (multiplet to quartet). These must be due to C-7 and C-8, in that order on chemical shift grounds. C-7, however, does not



carry the methoxyl group: decoupling of the methoxyl protons affects only the signal at 159.3 p.p.m. (quintet to doublet) which is assigned to C-5 by elimination of the other two oxygenated aromatics. The phenolic acetate group must therefore be placed at C-7. Decoupling of the aromatic proton gives results entirely consistent with its revised position at C-6. Three signals are affected: at 159.3 p.p.m. (quintet to quartet, C-5), at 154.5 p.p.m. (quartet to triplet, C-7) and at 108.8 p.p.m. (doublet to singlet, C-4a). C-8 and C-8a are unaffected. Finally, decoupling of the side-chain methylene bearing the aliphatic acetate group sharpens the signal at 110.2 p.p.m. (multiplet to quartet, C-8) and also allows the assignment of the aliphatic acetate carbonyl to the signal at 170.2 p.p.m. All the carbon-hydrogen couplings revealed by these experiments are summarised in Figure 3. Several two-bond couplings are resolvable; this has been previously noted in phenolic acetates<sup>12</sup>.

A revised structure (17) for LL-D253 $\alpha$  diacetate is therefore required and the <sup>13</sup>C spectral assignments for this structure are given in Table 2.

Our sample and an authentic sample<sup>13</sup> of LL-D253 $\alpha$  were carefully compared: they proved identical in all respects. The comparison included high resolution (300 MHz) proton n.m.r. spectroscopy of both samples. As LL-D253 $\alpha$  (16) is, for most practical purposes, insoluble in chloroform, the <sup>1</sup>H n.m.r. spectrum has only been previously reported<sup>3</sup>

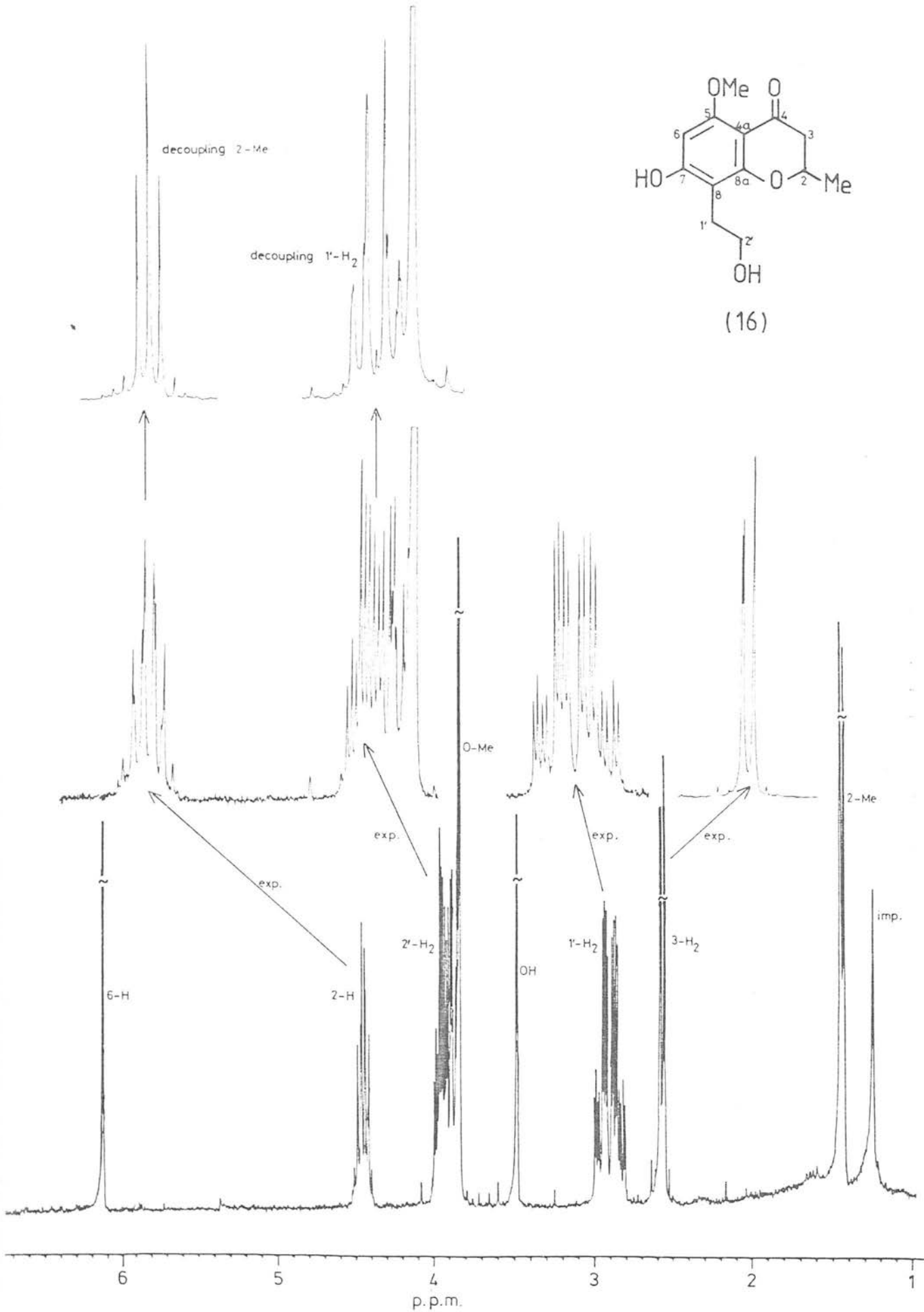
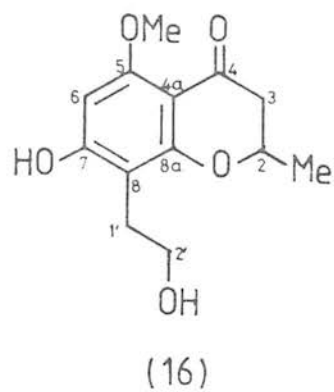


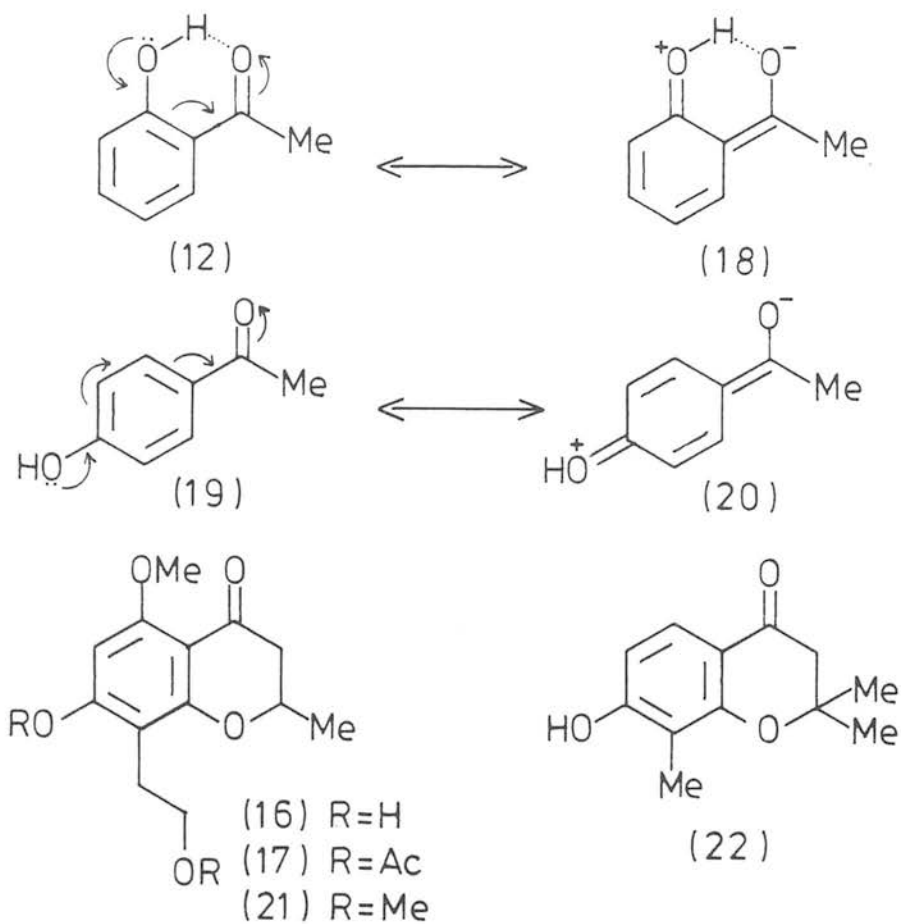
Figure 4 The 300 MHz  $^1\text{H}$  n.m.r. spectrum of LL-D253 $\alpha$  (in  $\text{CDCl}_3$ )

for a sample dissolved in  $d_4$ -methanol. This solvent has the disadvantage that any hydroxylic protons of the sample undergo exchange and do not give rise to meaningful signals. Our spectra were therefore obtained from extremely dilute solutions in  $CDCl_3$  using a Fourier-transform machine. Under these conditions, a chelated, phenolic, hydroxyl proton would be expected to give a sharp singlet above 10 p.p.m.; no such signal was present.

At this high field-strength (7.05 Tesla) several interesting features of the  $^1H$  n.m.r. spectrum become apparent (Figure 4 ). The H-2 and H-3 protons form an ABX type system, better seen on decoupling of the 2-methyl protons when the complex multiplet between 4.38 and 4.52 p.p.m. due to H-2 simplifies to the six-line X pattern ( $J_{AX} + J_{BX} = 15.4$  Hz,  $J_{AB} = 16.6$  Hz). Perhaps less predictable was the appearance of the signals arising from the two methylene groups of the hydroxyethyl side-chain. The sixteen-line signal between 2.79 and 3.00 p.p.m., due to the benzylic methylene group, appears to be composed of an AB quartet ( $J_{AB} = 15.3$  Hz) with further doublet of doublet splitting ( $J = 6.5, 3.0$  Hz or  $J = 7.9, 3.3$  Hz). Although the signal between 3.84 and 4.00 p.p.m., due to the hydroxyl-bearing methylene group, is not so well resolved because of coincident lines and the proximity of the methoxyl resonance, the same analysis appears to apply. Decoupling of the benzylic methylene protons gives an AB quartet ( $J_{AB} = 9.6$  Hz). This ABXY spin-system suggests that bond rotation of the hydroxyethyl side-chain

Table 3 Carbonyl infra-red absorptions<sup>20</sup> of some substituted acetophenones

Compound	Carbonyl Frequency (cm <sup>-1</sup> )
acetophenone	1685
2-hydroxyacetophenone	1635
3-hydroxyacetophenone	1670
4-hydroxyacetophenone	1640
2,6-dihydroxyacetophenone	1625
2,4-dihydroxyacetophenone	1630
2,4,6-trihydroxyacetophenone	1625
2-methoxyacetophenone	1670
3-methoxyacetophenone	1680
4-methoxyacetophenone	1670



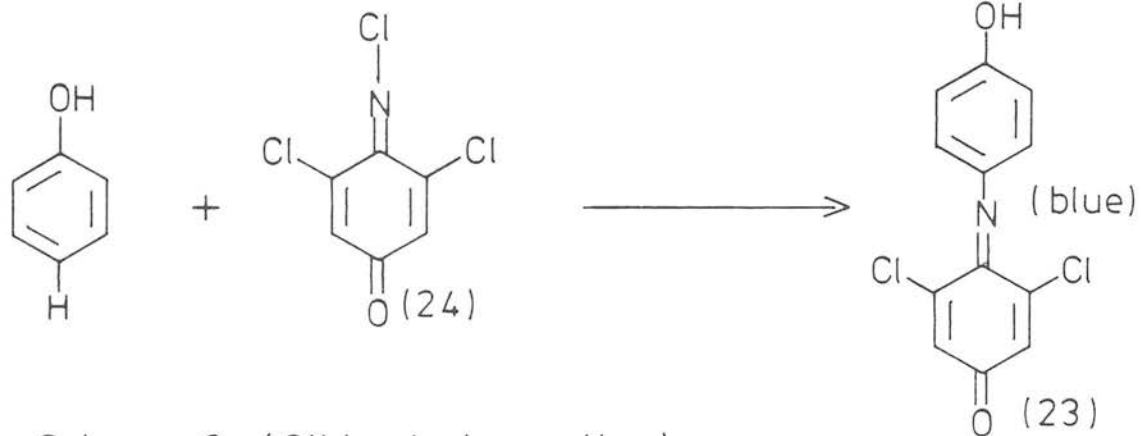
is restricted, probably by intramolecular hydrogen bonding. Support for this comes from the sharp hydroxyl resonance at 3.48 p.p.m. and from the proton n.m.r. spectrum of the diacetate (Figure 1 ) which shows simple triplets for the two side-chain methylene groups.

The evidence which led previous workers to adopt the *ortho*-hydroxycarbonyl structure (1) for LL-D253a must also be considered. This included the bathochromic and hyperchromic effects on the  $\pi$ - $\pi^*$  u.v. absorption of a sample in basic solution as opposed to one in methanol. As these effects mainly reflect the increased availability for conjugation of the phenolic oxygen electrons in the case of the phenolate anion<sup>14</sup>, there seems no *a priori* reason why the *para*-hydroxycarbonyl structure (16) should be excluded on these grounds.

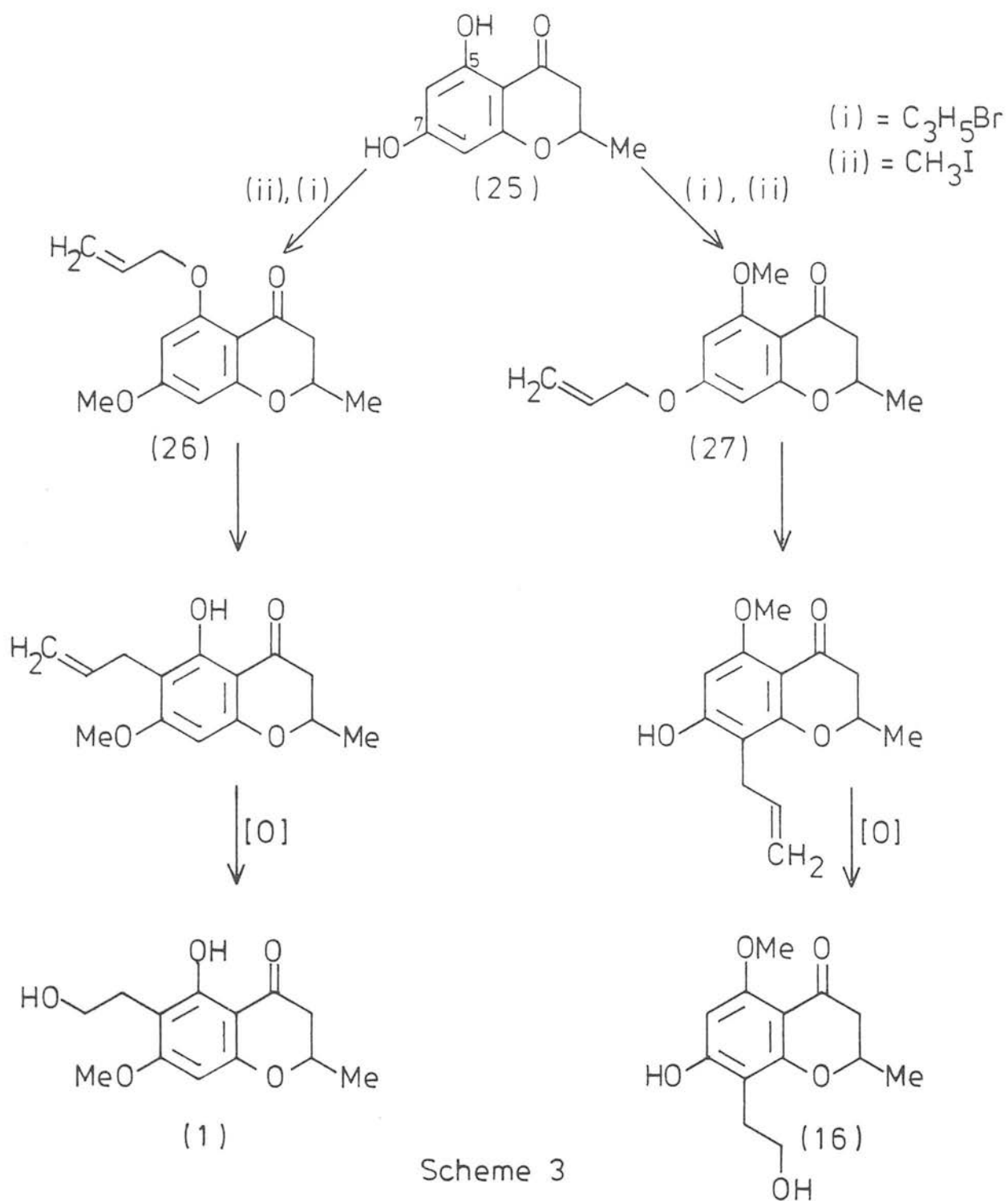
On the other hand, the infra-red data, cited in support of the original structure (1) and confirmed for our sample, are, at first sight, surprising. Carbonyl absorptions of around  $1640\text{ cm}^{-1}$  are frequently invoked<sup>15,16,17</sup> as additional evidence for chelation in support of *ortho*-hydroxycarbonyl structures, especially if the frequency rises on ether or ester formation. However, it appears that the low-frequency absorption of such a carbonyl, *e.g.* that in *ortho*-hydroxyacetophenone (12), is not due to the hydrogen-bonding itself. Rather, the hydrogen-bonding is said to stabilise a resonance form (18) thus reducing the double-bond character of the carbonyl group<sup>18,19</sup>.

The term "conjugate chelation" was coined to distinguish the effect from the much smaller shift produced on chelation by simple hydrogen-bonding<sup>19</sup>. A similar resonance form (20) can be drawn for *para*-hydroxyacetophenone (19) although this could not be stabilised by hydrogen-bonding. Examination of published i.r. spectra<sup>20</sup> of various acetophenones provides support for this suggestion (Table 3 ). The substitution of acetophenone with a hydroxyl group at either the *ortho* or *para* ring position produces a practically identical low-frequency shift (*ca.* 50 cm<sup>-1</sup>) of the carbonyl absorption. *Meta* substitution, as expected, produces a much smaller shift. Multiple substitution in the *ortho* and *para* positions has only a small additional effect, *i.e.* the shifts are not additive. In contrast, the carbonyl absorptions of the *ortho*, *meta* and *para* substituted methyl ethers are all around 1675 cm<sup>-1</sup>. While these data draw into question the role, if any, of hydrogen-bonding in stabilising the non-aromatic forms, *e.g.* (18), the main point is that the infra-red data on LL-D253 $\alpha$  (16), its diacetate (17) and its dimethyl ether (21) are not inconsistent with the revised structure (16) for LL-D253 $\alpha$ . A rather closer example of the effect of *para*-hydroxy substitution is found in 7-hydroxy-2,2,8-trimethyl-4-chromanone (22): the pyranone carbonyl resonance was reported<sup>21</sup> to occur at 1645 cm<sup>-1</sup> (nujol).





Scheme 2 (Gibbs test reaction)



Scheme 3

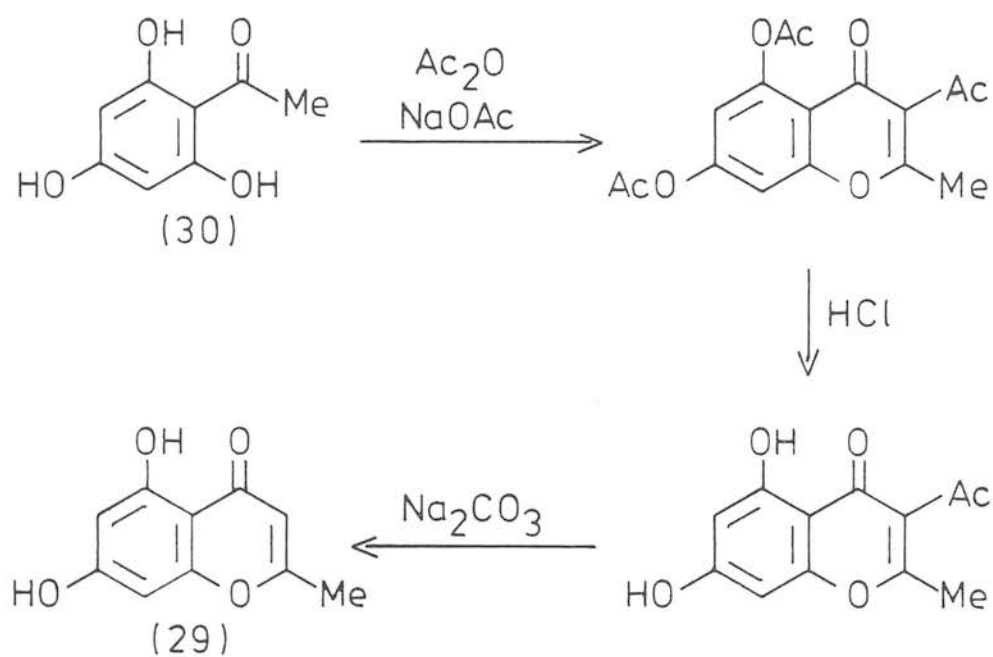
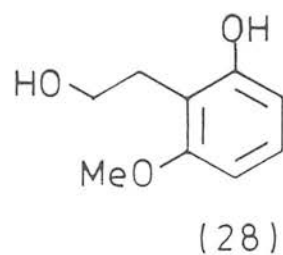
The final piece of evidence in favour of structure (1) is the positive Gibbs test. This is purported to show the presence of an unsubstituted CH *para* to a phenolic hydroxyl by the production of the blue indophenol (23) colouration when the phenol in question is added to a solution of 2,6,*N*-trichloro-*p*-benzoquinoneimine<sup>2</sup> (24) (Scheme 2). However, erroneous positive indications have been previously reported<sup>22,23</sup>: the Gibbs test alone is not sufficiently reliable to put the revised structure (16) in serious doubt. The test is best carried out in conjunction with a u.v. spectrophotometer to increase its reliability<sup>24</sup>.

Further evidence for the modified structure (16) for LL-D253 $\alpha$  was subsequently provided by total syntheses of both the original (1) and revised (16) structures; these are discussed in the following section.

### 3.3 Synthesis of LL-D253 $\alpha$ and Two of its Isomers

#### 3.3.1 Synthesis of LL-D253 $\alpha$

The wide range of synthetic approaches towards 4-chromanone compounds has been previously reviewed<sup>25,26</sup>. In the synthesis of the structures (1) and (16), the initial objective appeared to be 5,7-dihydroxy-2-methyl-4-chromanone (25). As *O*-alkylation of such a compound is known<sup>27</sup> to take place preferentially at the 7-position, selective mono-methylation or mono-allylation looked feasible. Further allylation or methylation would yield



Scheme 4

the isomeric structures, (26) and (27). Claisen rearrangement and oxidative cleavage of the *C*-allyl group would then allow the elaboration of the hydroxyethyl side-chain (Scheme 3), as exemplified in the published synthesis<sup>28</sup> of 2-(2-hydroxy-6-methoxyphenyl)ethanol (28).

Adopting this as the general strategy, a preparation of the starting chromanone (25) was first sought. A previous report<sup>29</sup> describes such a synthesis from phloroglucinol (2) and crotonic anhydride by the action of aluminium trichloride. No yield was quoted and attempts to reproduce this result using the stated conditions were unsuccessful, only starting material being recovered.

Attention was therefore turned to an alternative approach: reduction of the corresponding chromone species (29). This was easily prepared by a published procedure<sup>30</sup> (Scheme 4), using the Kostanecki-Robinson reaction<sup>31</sup>, from phloroacetophenone (30), itself obtained by a standard literature method<sup>32</sup>. Despite, however, the many encouraging precedents<sup>33</sup>, attempts to prepare 5,7-dihydroxy-2-methyl-4-chromanone (25) by catalytic hydrogenation of the chromone (29) met with only slight success. The use, in dry ethanol at room temperature, of either carbon-supported palladium or platinum oxide (Adams catalyst) at either low (1 atmosphere) or medium (10 atmospheres) pressures of hydrogen returned mostly starting chromone, although small proportions (10-15%) of the required chromanone were shown to be present by <sup>1</sup>H n.m.r. spectroscopy. Prolonged reaction times did not appear

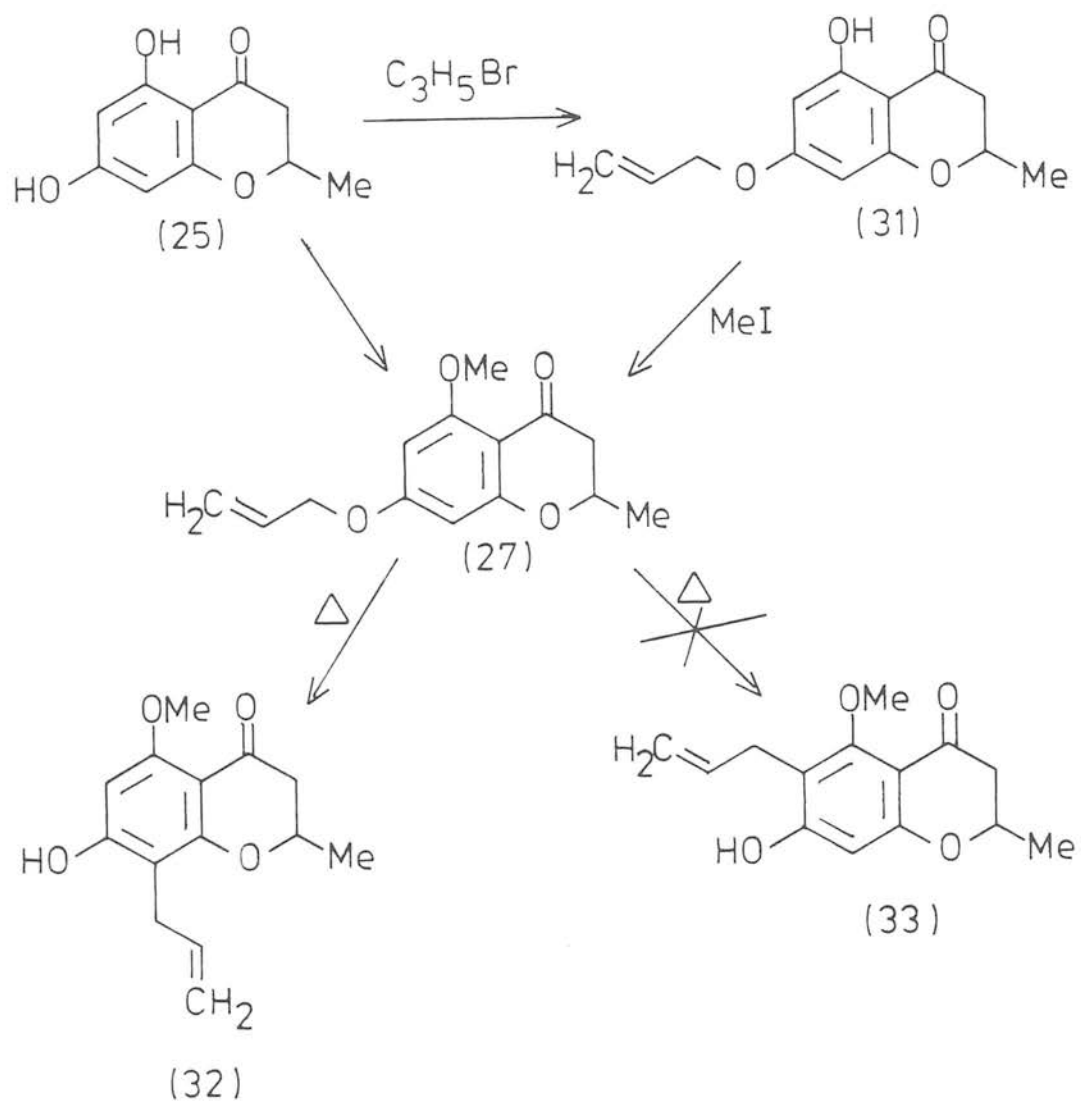
to increase the yield. Platinum oxide in acetic acid at low pressure of hydrogen gave a similar result.

Two non-catalytic reagents which have been reported as effective for the reduction of similar double bonds; namely, lithium in ammonia<sup>34</sup> and lithium aluminium hydride in tetrahydrofuran<sup>35</sup>, were also applied to 5,7-dihydroxy-2-methylchromone (29) but without success. As adjusting the conditions of the Friedel-Crafts reaction<sup>29</sup> later gave the chromanone (25) directly in passable yield, further efforts to reduce the chromone (29) were abandoned. A very recent publication, however, reports its apparently easy catalytic reduction using carbon-supported palladium in ethyl acetate<sup>36</sup>.

Using the modified procedure described in the experimental section, the Friedel-Crafts reaction of phloroglucinol and crotonic anhydride afforded 5,7-dihydroxy-2-methyl-4-chromanone (25) in 36% yield, (similar to the yield obtained elsewhere<sup>37</sup> using  $\text{BF}_3$  gas and crotonic anhydride). Most of the compounds which were subsequently prepared from this starting chromanone, in the course of the syntheses of the original (1) and revised (16) structures for LL-D253 $\alpha$ , do not appear to have been previously described. Full chemical characterisation data for these appear in the experimental section.

Reaction of the dihydroxychromanone (25) with one equivalent of allyl bromide in the presence of potassium carbonate in acetone gave the mono-allyl ether (31) in



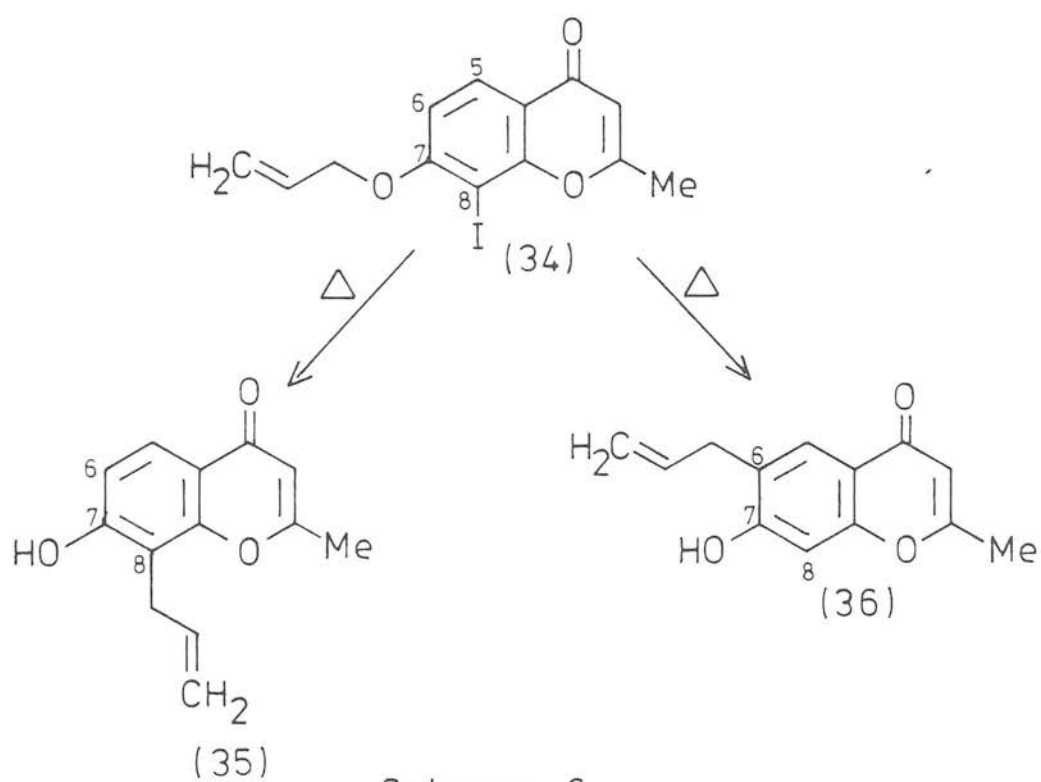


Scheme 5

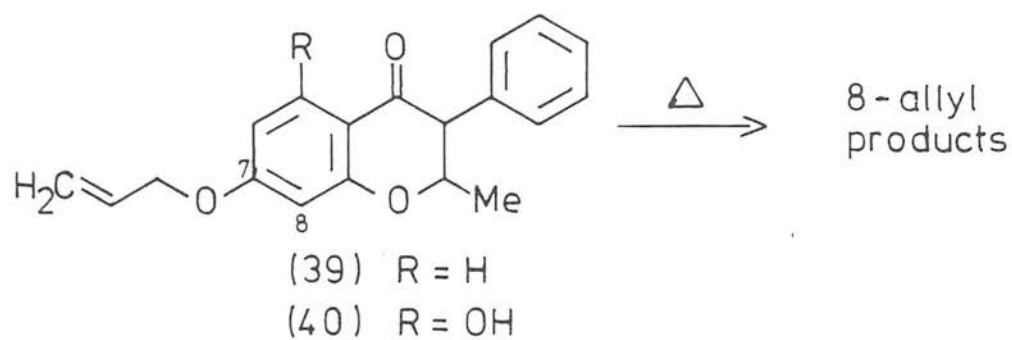
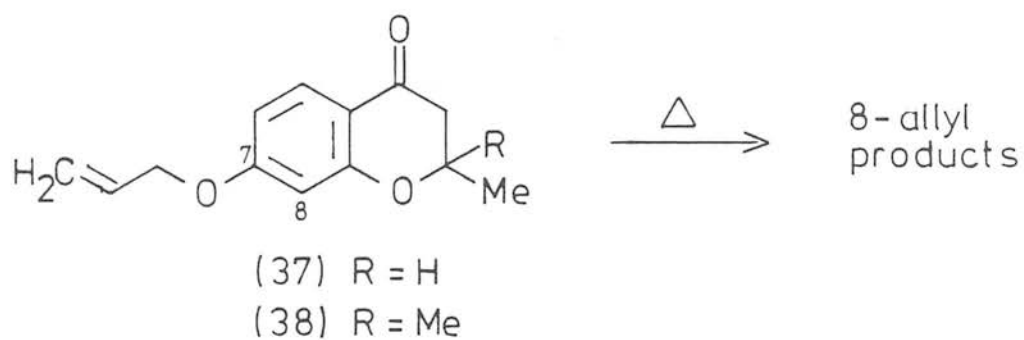
64% yield. As expected, alkylation occurred only on the non-chelated 7-hydroxyl group, as shown by the presence of a sharp singlet at 12.04 p.p.m. in the  $^1\text{H}$  n.m.r. spectrum. This compound, unlike the dihydroxychromanone (25) itself but in common with all its other 7-alkylated derivatives, was readily soluble in chloroform, providing further circumstantial evidence against structure (1) for LL-D253 $\alpha$ .

Treatment of the mono-allyl ether (31) with methyl iodide afforded 7-allyloxy-5-methoxy-2-methyl-4-chromanone (27) in 81% yield. This compound was subsequently prepared in a "one-pot" procedure from the dihydroxychromanone (25). The overall yield (50%) was not improved but only one work-up was required.

The Claisen rearrangement of this diether (27) was best carried out by heating it at 200°C *in vacuo*. (Boiling in *N,N*-dimethylaniline gave only a 32% yield of the *C*-allyl phenol.) In principle, the rearrangement could have proceeded in either direction to yield the isomeric products, (32) and (33). As anticipated though, only one of these products was actually obtained (Scheme 5). It has been previously pointed out<sup>38</sup> that there is a general tendency for alkyl groups on benzopyrone nuclei to adopt the 8-position, when given the choice between that and the 6-position. This is seen in the Wessely-Moser rearrangement of 6-alkylchromones or flavones which also carry a hydroxy or alkoxy substituent at the 5-position: when



Scheme 6



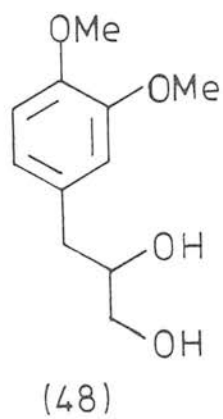
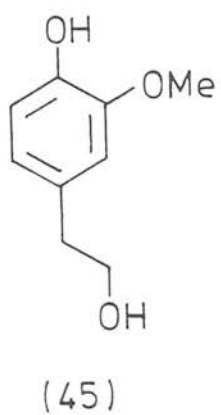
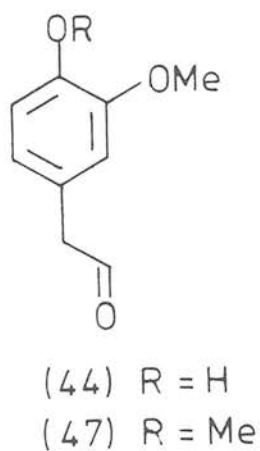
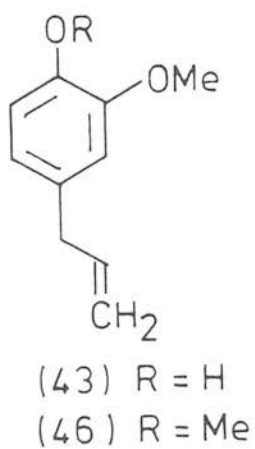
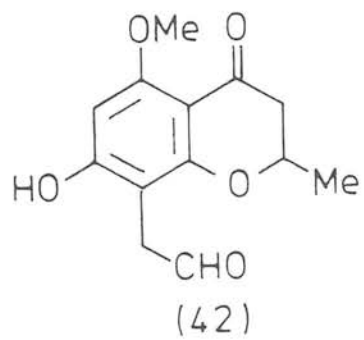
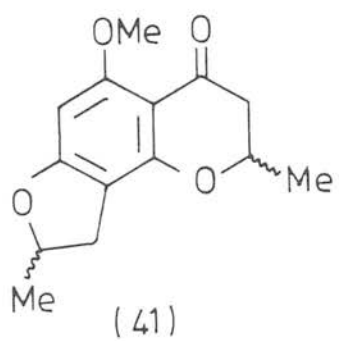


heated with (typically) hydriodic acid, pyrone ring opening and recyclisation to the opposite site usually gives a predominance of the 8-alkyl isomer.

Claisen migration of 7-allyloxychromones resulted in the exclusive formation of the 8-allyl-7-hydroxy chromones unless the 8-position was already substituted<sup>39</sup>. Even the 8-iodochromone (34) yielded equal amounts of the 8-allyl derivative (35) along with the required 6-allyl compound (36) (Scheme 6). Precedent in the chromanone series is rather scarcer, but what is available also follows this general trend. The 7-allyloxychromanones, (37) and (38), and the 7-allyloxyisoflavanones, (39) and (40), all reportedly gave their 8-allyl-7-hydroxy products on heating<sup>40</sup>.

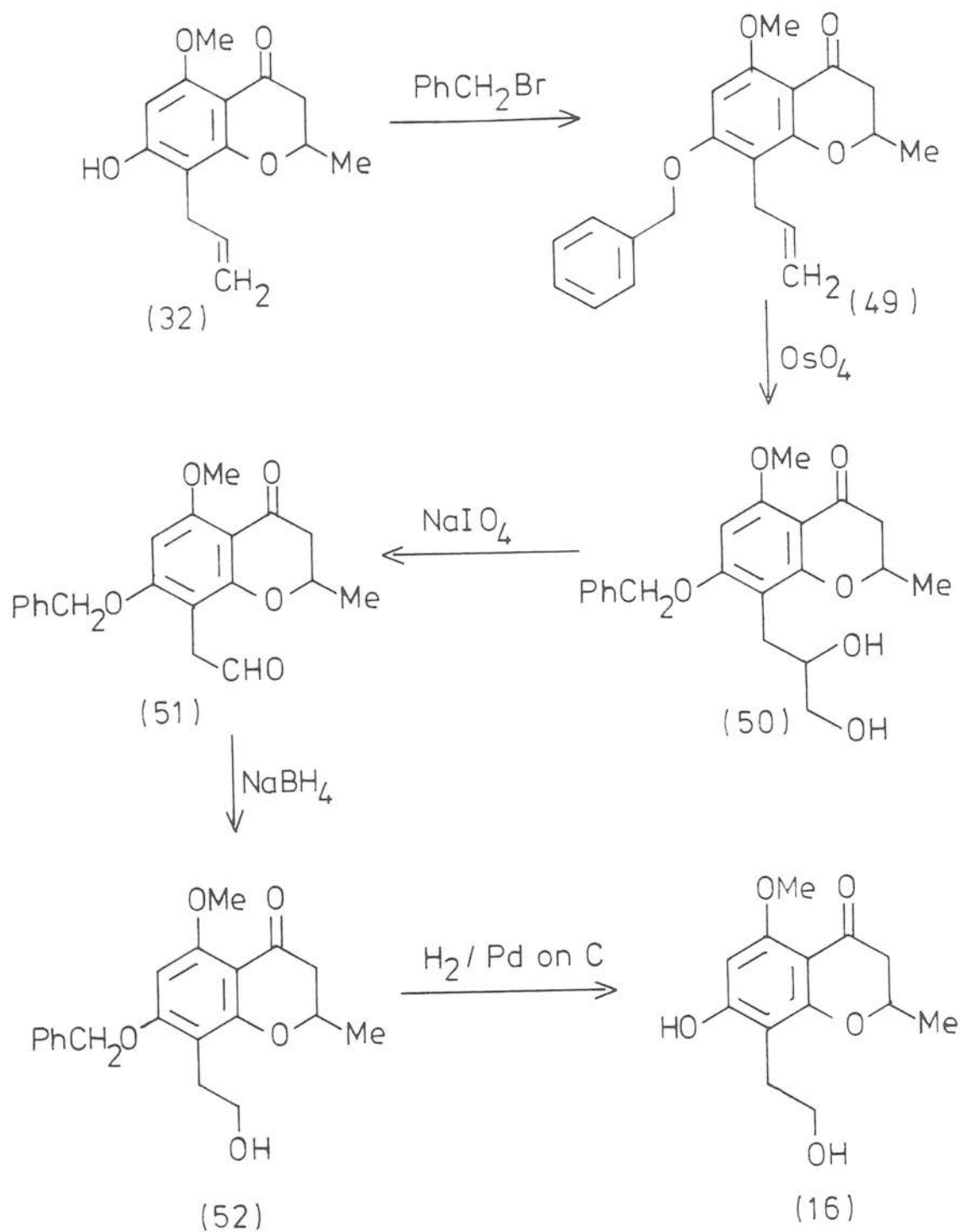
In view of this consistent background, the *C*-allyl phenol, obtained in 68% yield from the pyrolysis of the diether (27) was designated 8-allyl-7-hydroxy-5-methoxy-2-methyl-4-chromanone (32). The *C*-allyl group was identified by the benzylic methylene signal in the <sup>1</sup>H n.m.r. spectrum at 3.30 p.p.m., showing an allylic coupling pattern. From the infra-red and <sup>1</sup>H n.m.r. spectra, and from the insolubility of the compound in chloroform, the presence of an unchelated phenolic hydroxyl was evident.

This pyrolysis also produced, in minor quantities, a second compound, at first mistaken for the starting material (27) with which it co-developed on t.l.c. The <sup>1</sup>H n.m.r. spectrum of this material contains signals very similar, and in addition, to those arising from the -O-CHR-CH<sub>3</sub> fragment



of the chromanone nucleus. Also observed are two single-proton signals at 2.64 and 3.18 p.p.m. which each display, *inter alia*, a geminal (15 Hz) coupling. All these new signals are present as slightly separated pairs suggesting a diastereoisomeric mixture. On this basis the compound is tentatively identified as 5-methoxy-2,8-dimethyl-2,3,8,9-tetrahydro-4*H*-furo[2,3-*h*]-1-benzopyran-4-one (41). It is presumably obtained from the initial product (32) by intramolecular addition.

An attempt was next made to reach the aldehyde (42) directly from the 8-allylchromanone (32) by ozonolysis (in methanol at -78°C) followed by the reductive work-up of the ozonide using dimethylsulphide<sup>41</sup>. This was unsuccessful: an intractable mixture of products was obtained. At this point, in order to establish suitable conditions for the cleavage of the allyl group, a series of experiments on a model compound, eugenol (43), were conducted. Although the aldehyde (44) has been prepared<sup>42</sup> by the ozonolysis of eugenol, it is known to be rather unstable. For this reason, the reaction mixture obtained from the ozonolysis of eugenol (in methanol at -78°C) was treated with sodium borohydride before it was allowed to warm to room temperature. On attempted isolation of the target alcohol (45), however, only what appeared to be a decomposition product was actually obtained: the proton n.m.r. spectrum showed no aromatic signals. A similar situation prevailed when the same procedure was applied to eugenol methyl-ether (46).



Scheme 7

The use of osmium tetroxide and sodium periodate was thus investigated. Using only a catalytic quantity of  $\text{OsO}_4$  and an excess of periodate, in a procedure successfully applied<sup>43</sup> to a similar allyl compound, returned mostly starting material when eugenol was the substrate. Increasing the amount of  $\text{OsO}_4$  and using eugenol methyl-ether gave a better result. Finally, the aldehyde (47) was obtained in quantitative yield by the initial application of one equivalent of  $\text{OsO}_4$  to eugenol methyl-ether followed by periodate cleavage of the intermediate diol (48).

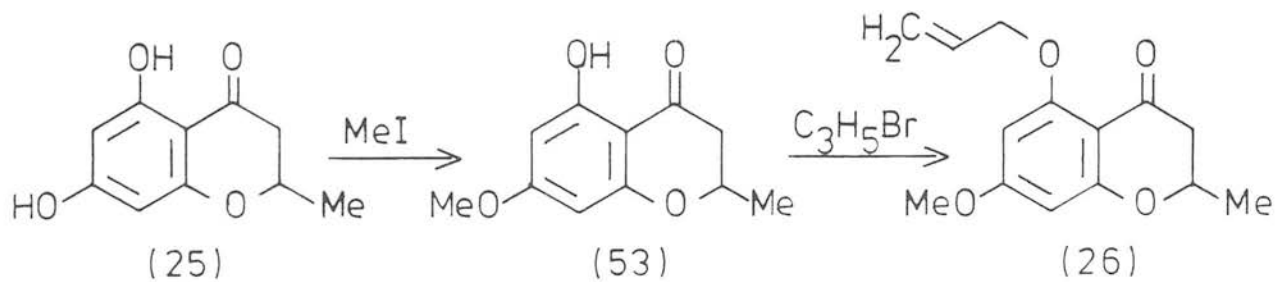
(These products were identified by proton n.m.r. spectroscopy and mass spectrometry but were not fully characterised. The reaction conditions used were the same as those later described for the cleavage of the *C*-allyl chromanones.)

In the light of this result, it was decided to protect the phenolic hydroxyl of the 8-allylchromanone (32) before attempting the oxidative cleavage of the allyl group (Scheme 7). Benzylolation by benzyl bromide and potassium carbonate in acetone proceeded in quantitative yield and the benzyl ether (49) was treated with one equivalent of osmium tetroxide. The osmate ester was cleaved with sodium metabisulphite<sup>44</sup> and the diol (50) was isolated as a diastereomeric mixture (94%) which was characterised without separation. Many of the signals in the 360 MHz proton n.m.r. spectrum of this compound therefore appeared as slightly separated pairs. Both methylene groups of the dihydroxypropyl moiety of each

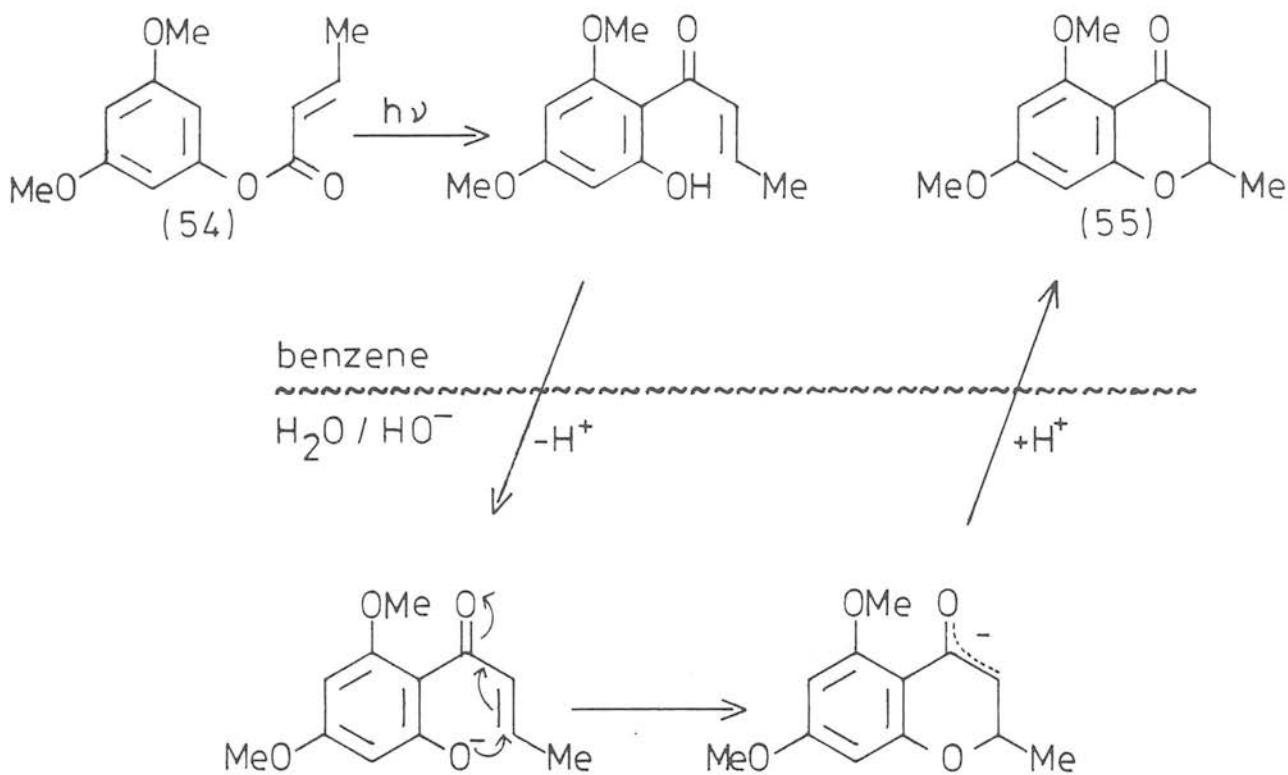
diastereoisomer gave an AB of ABX pattern through coupling to the central proton. Whether the chemical non-equivalence of the two hydrogens of each methylene group arises solely from the proximity of the assymmetric carbon atom, or whether hydrogen-bonding contributes by restricting the motion of the dihydroxypropyl group, is not clear.

Treatment of the diol (50) with sodium periodate<sup>45</sup> gave a quantitative yield of the required aldehyde (51). This was identified by its infra-red carbonyl band at  $1724\text{ cm}^{-1}$ , by the low-field triplet (9.60 p.p.m.) in its  $^1\text{H}$  n.m.r. spectrum and by the  $^{13}\text{C}$  n.m.r. signal at 199.4 p.p.m. The same compound was later obtained (81%) by the use of pyridinium chlorochromate<sup>46</sup> on the benzyl ether of natural LL-D253 $\alpha$ . Attempts to rigourously purify the aldehyde (51) by recrystallisation resulted in decomposition.

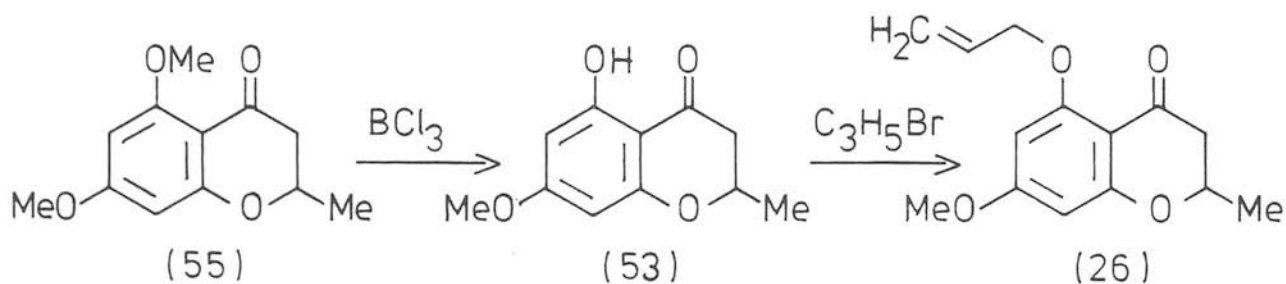
The selective reduction of aldehyde carbonyls in the presence of ketone carbonyls by using sodium borohydride in tetrahydrofuran has been previously reported<sup>47</sup>. This was found to be an effective procedure for the selective reduction of the aldehyde (51) to the hydroxyethyl species (52) which was thereby obtained in 72% yield. Comparison of this compound with the benzyl ether of naturally occurring LL-D253 $\alpha$  showed that they were identical in all measured respects.



Scheme 8



Scheme 9



Scheme 10

(±)LL-D253α itself was obtained by catalytic hydrogenolysis of the benzyl protecting group. Careful monitoring of the progress of the reaction was required to avoid further reduction of the product: the best yield obtained was 63% (69% allowing for recovered starting material). Again, the chemical and physical properties of the synthetic material matched those of the natural product, the identity of which is therefore confirmed as 7-hydroxy-8-(2-hydroxyethyl)-5-methoxyl-2-methyl-4-chromanone (16). The overall yield of (±)LL-D253α from 5,7-dihydroxy-2-methyl-4-chromanone (25) was an acceptable 16%.

### 3.3.2 Synthesis of Two Isomers of LL-D253α

Synthesis of the original structure (1) was next investigated. 5-Allyloxy-7-methoxy-2-methyl-4-chromanone (26) was first prepared in 62% yield by a "one-pot" procedure; treating 5,7-dihydroxy-2-methyl-4-chromanone (25) initially with one equivalent of methyl iodide and subsequently with an excess of allyl bromide (Scheme 8). The product was characterised and shown to be different from the isomeric diether (27) prepared as an intermediate in the synthetic route to LL-D253α.

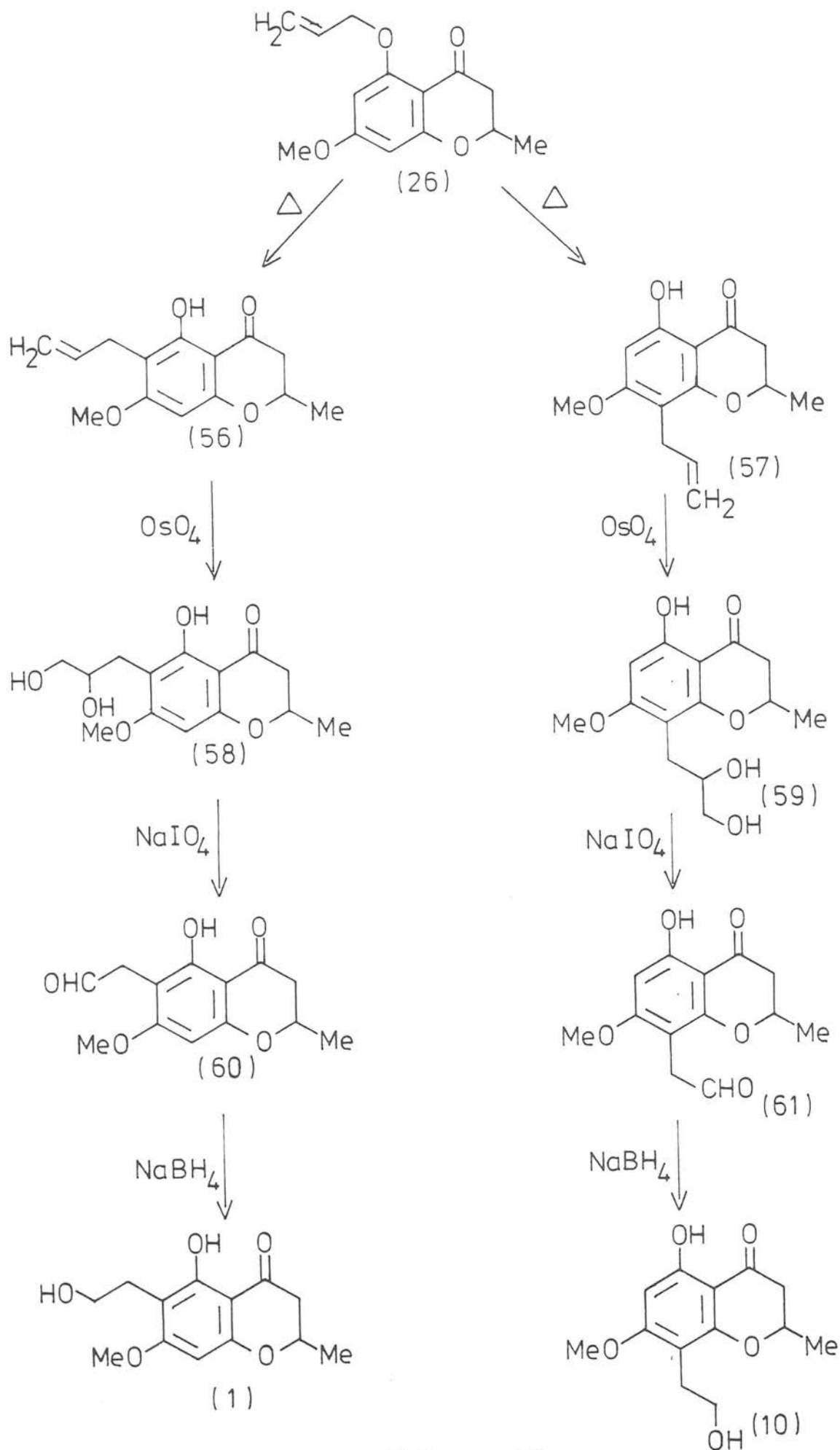
Although the compound first formed by this procedure, 5-hydroxy-7-methoxy-2-methyl-4-chromanone (53), was not isolated in this case, it was later obtained by a different route. While this work was in progress, a report<sup>48</sup> appeared describing a high-yielding preparation of



4-chromanones by a photo-Fries rearrangement of substituted aryl acrylates in a two-phase (benzene:aqueous-hydroxide) system. This procedure (Scheme 9) was therefore applied to 3,5-dimethoxyphenyl crotonate (54), itself prepared from 3,5-dimethoxyphenol and crotonyl chloride by a method previously described<sup>49</sup> for the synthesis of other phenyl crotonates.

The main advantage of this photochemical chromanone preparation was the easy isolation of the product, in this case the known<sup>50</sup> compound, 5,7-dimethoxy-2-methyl-4-chromanone (55). Unfortunately the best yield obtained was only 21% so the procedure did not prove as useful as had been initially hoped. Nevertheless, treatment of the dimethyl ether (55) with boron trichloride, a useful reagent for the selective demethylation of methoxyl groups *ortho* to carbonyls<sup>51</sup>, gave the 5-hydroxy compound (53) in 96% yield. The proton n.m.r. spectrum showed the sharp low-field signal (12.13 p.p.m.) characteristic of such a chelated phenol, confirming that it was indeed the 5-methoxyl group which had been demethylated. Allylation as before then gave the required diether (26) in 62% yield (Scheme 10). This second route, *via* the dimethyl ether (55), suffers from the low yield of the photochemical step but this reaction probably deserves further study. Much better yields of similar compounds were quoted in the original report<sup>48</sup> although few experimental details were given.

Claisen rearrangement of 5-allyloxy-7-methoxy-2-methyl-4-chromanone (26) on a small (50 mg) scale, at 200°C *in vacuo*,



Scheme 11

proceeded cleanly and quickly to give a quantitative yield of a single *C*-allyl compound, later identified as 6-allyl-5-hydroxy-7-methoxy-2-methyl-4-chromanone (56). This rearrangement occurred more readily than the corresponding rearrangement of the 7-allyloxy isomer (27): a shorter period of heating was required. There was also no sign of subsequent addition of the phenolic hydroxyl across the *C*-allyl double bond, probably owing to the lower reactivity of chelated hydroxyls. However, when this reaction was scaled up, using all available substrate, the sealed tube cracked under heating and admitted air. Examination of the contents now revealed the presence of two products. These were separated, with some difficulty, by repeated thin layer chromatography. The major product, obtained on this occasion in 46% yield, proved to be the same as that obtained in the small-scale experiment. It was accompanied by a minor (25% yield) component which displayed virtually identical spectroscopic characteristics. <sup>1</sup>H n.m.r. spectra of both compounds included the signals of an aromatic *C*-allyl group and of a chelated phenolic hydroxyl. Although the two products were confidently identified as 6-allyl-5-hydroxy-7-methoxy-2-methyl-4-chromanone (56) and its 8-allyl isomer (57), at this stage it could not be said for certain which was which. Spectroscopic studies on the hydroxyethyl derivatives, however, later established that the major and minor products were the 6-allyl (56) and 8-allyl (57) compounds respectively.

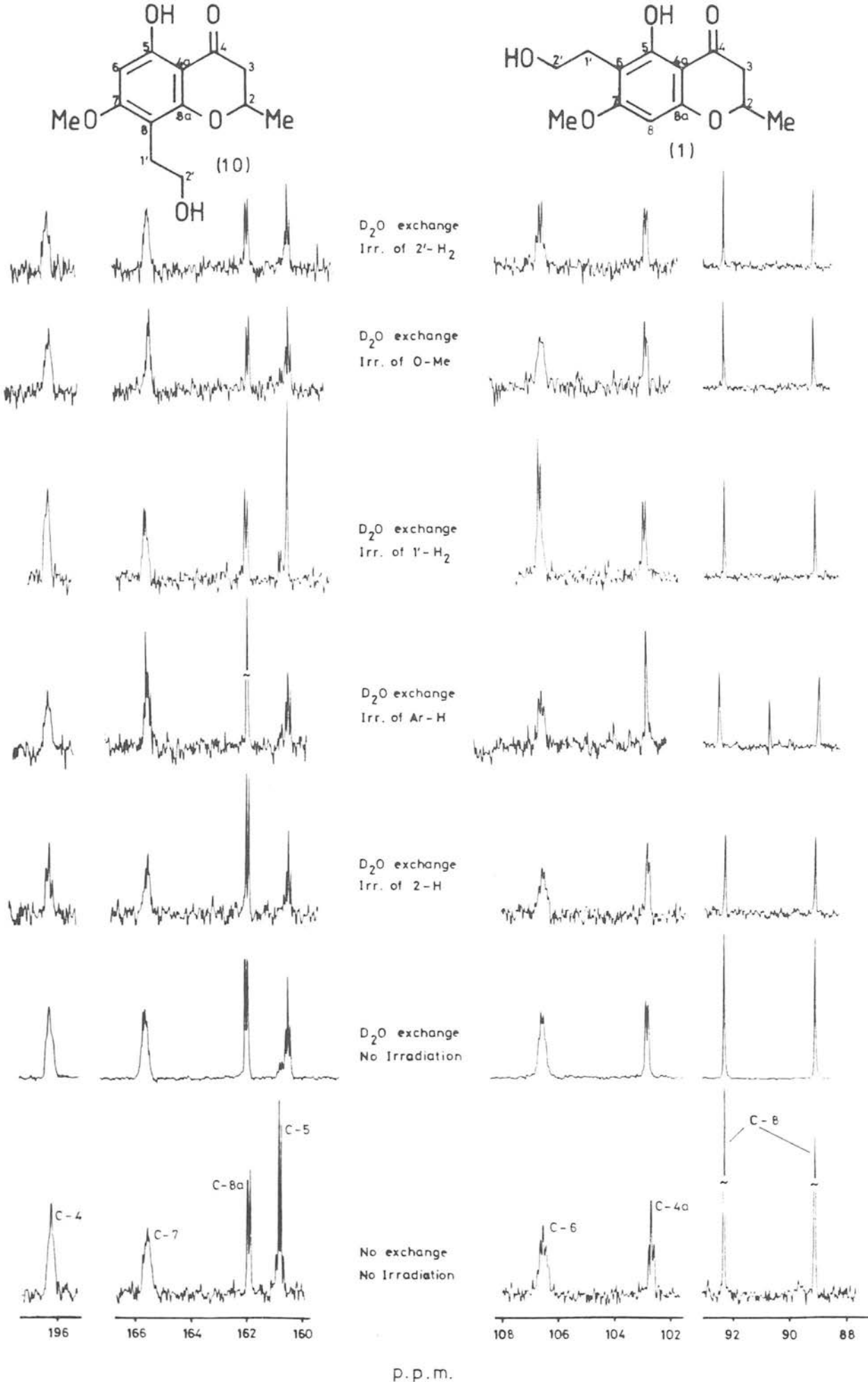


Figure 5

The low-field region of the fully  $^1\text{H}$ -coupled 50 MHz  $^{13}\text{C}$  n.m.r. spectrum of 5-hydroxy-6-(2-hydroxyethyl)-7-methoxy-2-methyl-4-chromanone (1) and the results of selective  $^1\text{H}$ -decoupling experiments (in  $\text{CDCl}_3$ ).

Both of these rearrangement products were subjected to osmium tetroxide oxidation to the vicinal diols, (58) and (59), periodate cleavage to the aldehydes, (60) and (61), and borohydride reduction to the hydroxyethyl species, (1) and (10), (Scheme 11), as in the synthesis of LL-D253 $\alpha$ . Prior protection of the phenol function proved unnecessary. All the intermediate species were isolated and characterised: here again, the aldehydes were sensitive to handling.

### 3.3.3 Identification of the Two Isomers

Figure 5 shows the low-field region of the fully proton coupled  $^{13}\text{C}$  n.m.r. spectrum of the hydroxyethyl species obtained from the major isomer produced by the Claisen rearrangement of the 5-allyl ether (26). The effects of adding  $\text{D}_2\text{O}$  and the results of a series of selective proton-decouplings are also shown. Three signals are noticeably modified by the addition of  $\text{D}_2\text{O}$  including the two due to the non-protonated, non-oxygenated pair of aromatic carbons at 102.7 and 106.6 p.p.m. which sharpen, respectively, to a doublet (from a triplet) and a quartet (from a multiplet).

The only other signal affected is that at 160.8 p.p.m. which simplifies from a quartet to a triplet and also displays a significant (*ca.* 0.2 p.p.m.) upfield shift, revealed by the presence of a residual quartet signal due to incomplete exchange. This signal is assigned to C-5 which is known, from the proton n.m.r. spectrum, to carry the

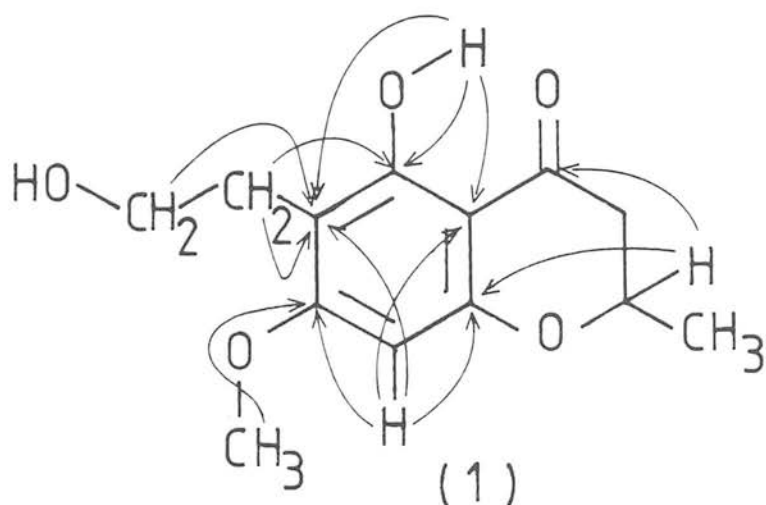


Figure 6 Long-range  $^1\text{H}$ - $^{13}\text{C}$  couplings in 5-hydroxy-6-(2-hydroxyethyl)-7-methoxy-2-methyl-4-chromanone (1) identified by selective  $^1\text{H}$ -decoupling experiments (see Figure 5).

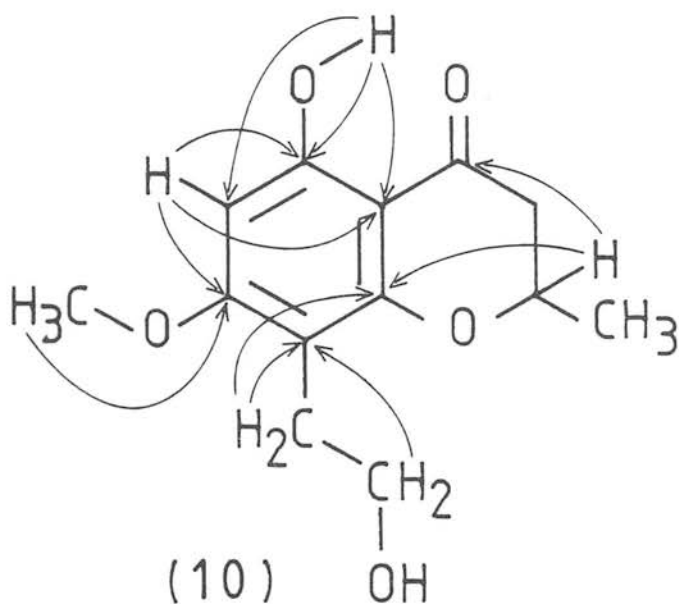


Figure 8 Long-range  $^1\text{H}$ - $^{13}\text{C}$  couplings in 5-hydroxy-8-(2-hydroxyethyl)-7-methoxy-2-methyl-4-chromanone (10) identified by selective  $^1\text{H}$ -decoupling experiments (see Figure 7).

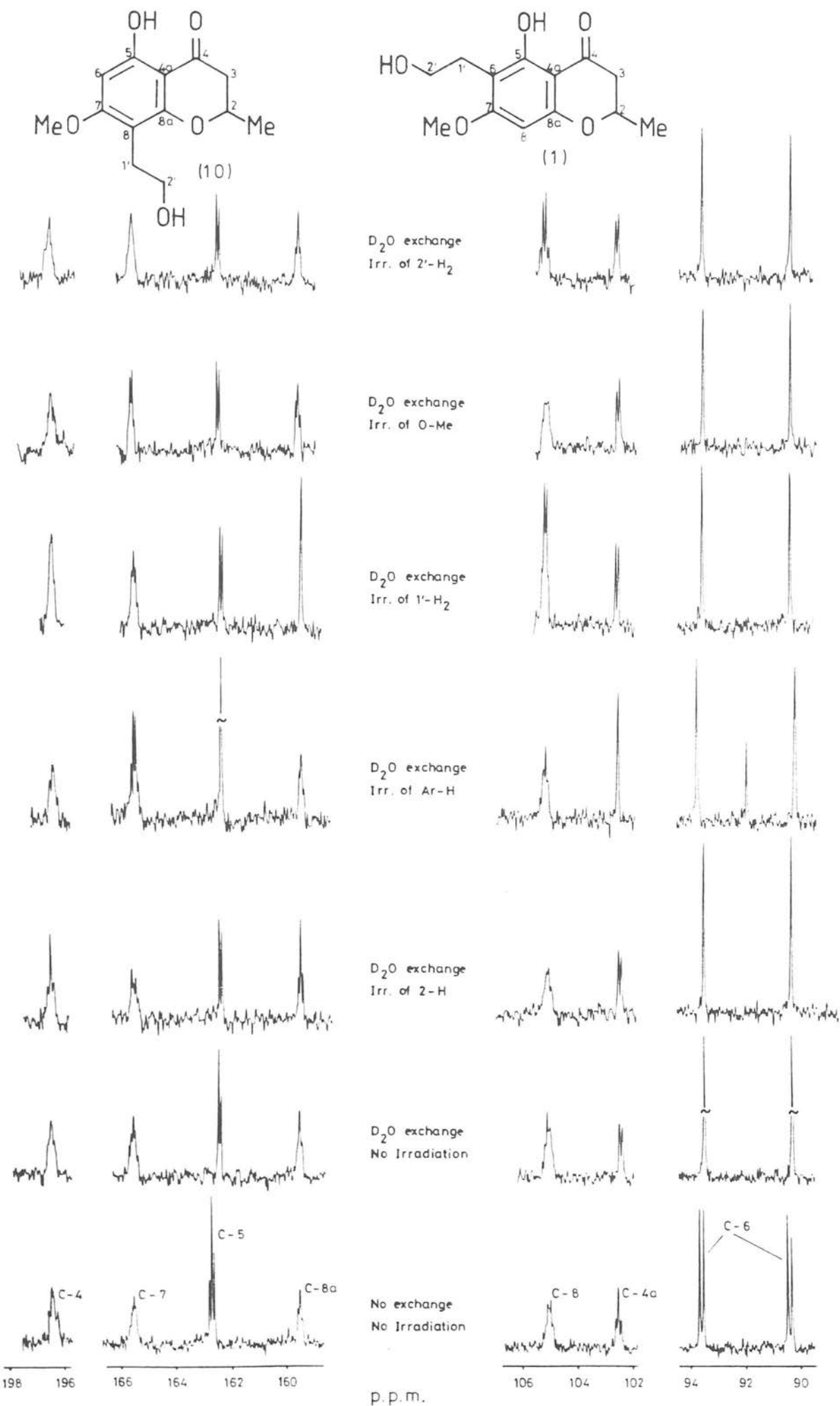


Figure 7

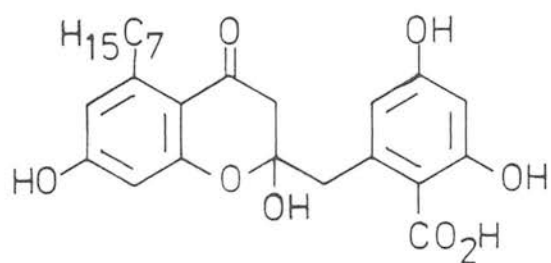
The low-field region of the fully  $^1\text{H}$ -coupled 50 MHz  $^{13}\text{C}$  n.m.r. spectrum of 5-hydroxy-8-(2-hydroxyethyl)-7-methoxy-2-methyl-4-chromanone (10) and the results of selective  $^1\text{H}$ -decoupling experiments (in  $\text{CDCl}_3$ ).

aromatic hydroxyl group. As the signal of the proton-bearing aromatic carbon is unaffected by D<sub>2</sub>O exchange, this carbon must be relatively remote, *i.e.* at the 8-position. Confirmation is available by decoupling H-2: the only aromatic signal sharpened is the doublet at 161.9 p.p.m. which is thus assigned to C-8a. Decoupling the aromatic proton collapses this same signal to a sharp singlet. No other decoupling has a significant effect on the C-8a signal. Decoupling the benzylic methylene hydrogens simplifies the C-5 triplet to a singlet and markedly sharpens the resonance at 106.6 p.p.m., thereby assigned to C-6.

The above observations effectively determine the structure (1) for this compound; the full extent of the carbon-hydrogen couplings revealed by these experiments is shown in Figure 6. This then, is the compound possessing the structure originally proposed<sup>1,3</sup> for LL-D253α: its physical properties and spectral characteristics are very different from the natural product. In particular, it is readily soluble in chloroform and its proton n.m.r. spectrum includes a sharp, exchangeable singlet at 12.13 p.p.m. due to the chelated hydroxyl. The best overall yield from 5,7-dihydroxy-2-methyl-4-chromanone (25) was 44%.

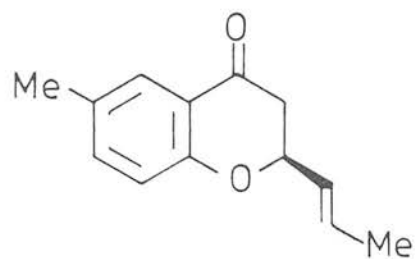
A similar series of proton-decouplings was carried out on the hydroxyethyl derivative obtained from the minor product of the Claisen rearrangement (Figure 7). This time the protonated aromatic carbon signal does display a





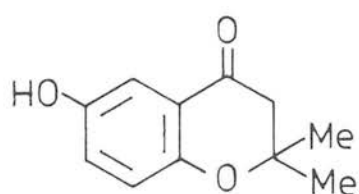
protosiphulin

from *Siphula ceratites*

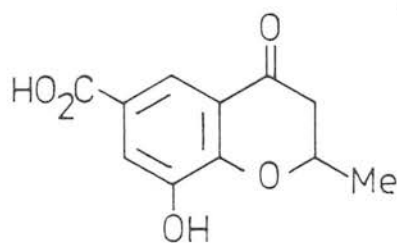


myrochromanone

from *Myrothecium roridum*

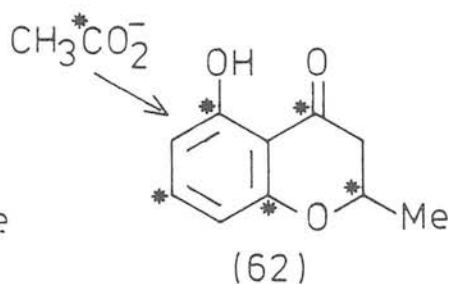


from *Panus conchatus*



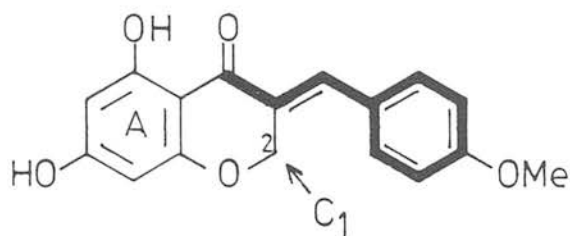
rosellinic acid

from *Rosellinia necatrix*

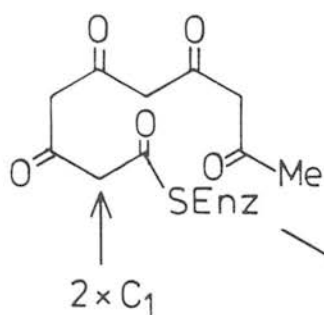
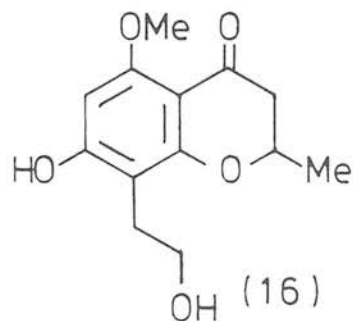


from *Daldinia concentrica*

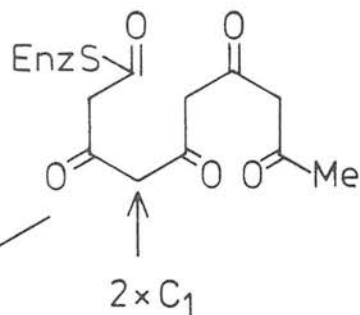
Figure 9 Some fungal 4-chromanones



(63) eucomin



or



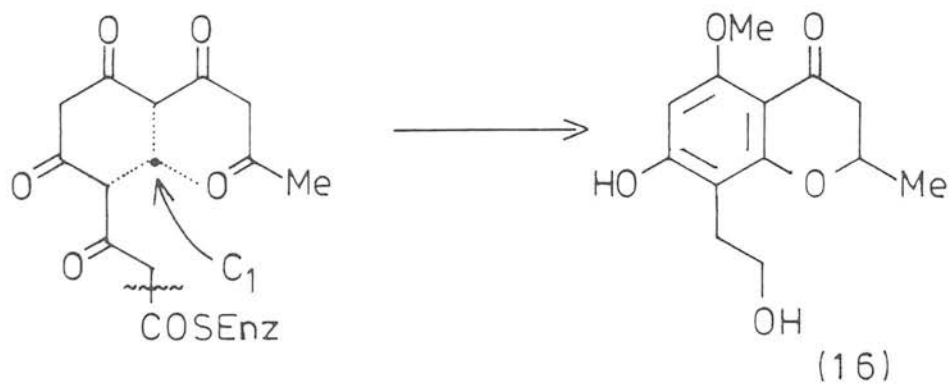
(16)

Scheme 12

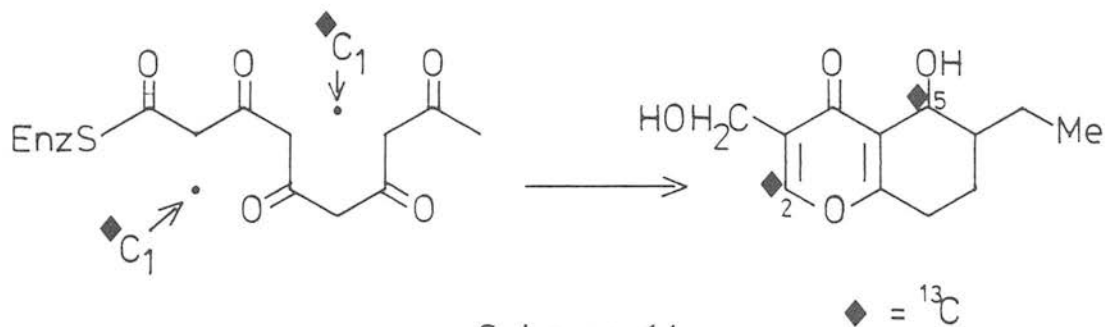
coupling, lost on D<sub>2</sub>O exchange, to the phenolic proton. In addition, the only aromatic signal (at 159.5 p.p.m.) affected by the decoupling of H-2 is also affected by the decoupling of the benzylic hydrogens but not by decoupling of the aromatic proton. Figure 8 shows a summary of the long-range carbon-hydrogen interactions. This compound is therefore independently shown to be 5-hydroxy-8-(2-hydroxyethyl)-7-methoxy-2-methyl-4-chromanone (10). The overall yield from the starting chromanone (25) was 8% although, given the circumstances of the Claisen rearrangement step, the figure is fairly meaningless. It would be interesting to discover whether or not a true *para* rearrangement occurred to give the 8-allyl isomer (57) from the 5-allyl ether (26). It is conceivable that opening of the pyranone ring took place followed by closure in the opposite sense to give the 8-allyl isomer from the initial 6-allyl product, *cf.* the Wessely-Moser rearrangement<sup>5</sup> of chromones. Perhaps deuterium labelling could reveal whether or not inversion of the allyl group occurs during the formation of the *para* product.

### 3.4 Background Information - Biosynthesis

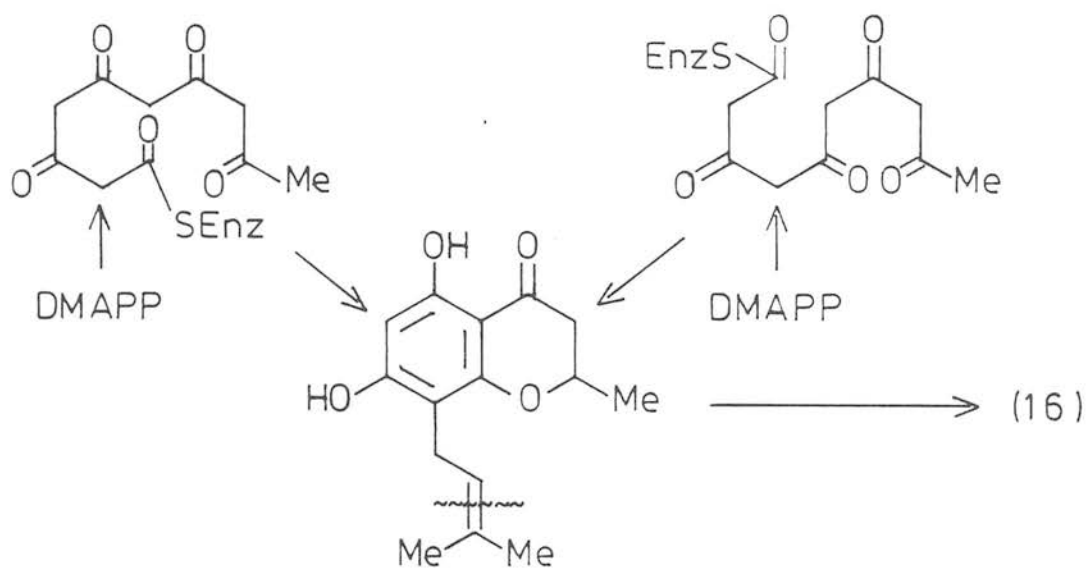
Naturally occurring 4-chromanones, as opposed to the related flavanones, appear to comprise a rather small group of compounds<sup>52</sup>. Only a few of these are fungal metabolites<sup>53</sup>; some examples are shown in Figure 9.



Scheme 13



Scheme 14



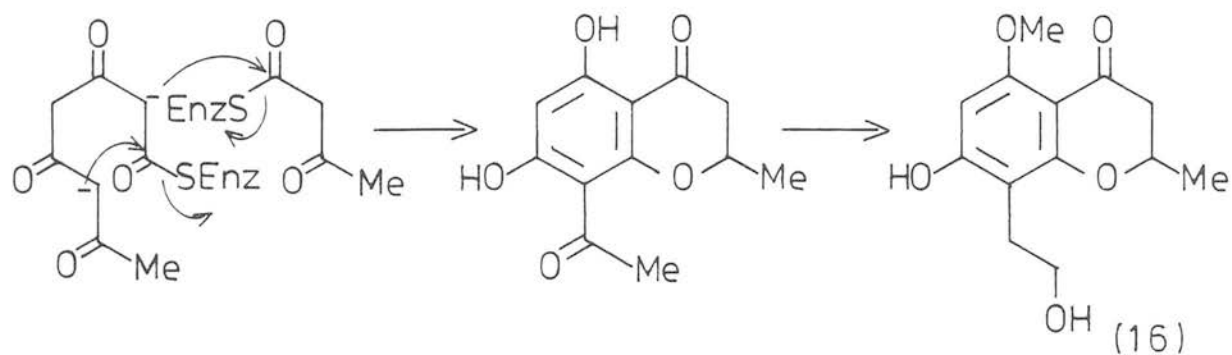
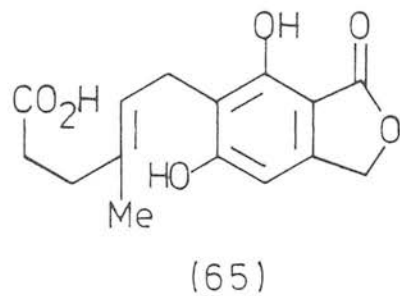
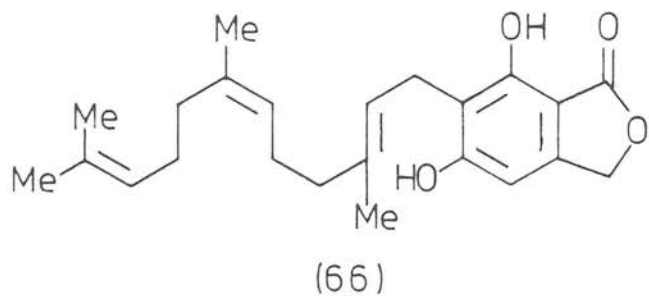
Scheme 15

Biosynthetic studies of 4-chromanones are hence very scarce. Cultures of the fungus, *Daldinia concentrica*, fed [1-<sup>14</sup>C]acetate, produced 5-hydroxy-2-methyl-4-chromanone (62) with the labelling pattern shown<sup>29</sup>. The biosynthesis of a plant product, eucomin (63), produced by *Eucomis bicolor* (Liliaceae) has also been investigated with <sup>14</sup>C-labelled precursors<sup>54</sup>. Phenylalanine was incorporated intact and methionine appeared to label C-2. Acetate labelled the aromatic ring A.

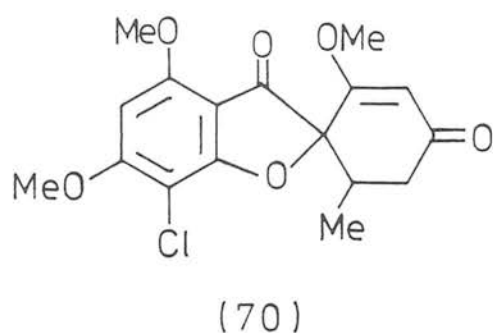
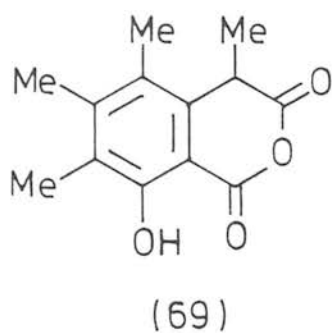
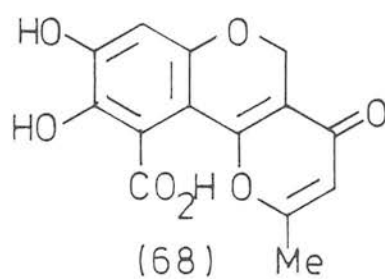
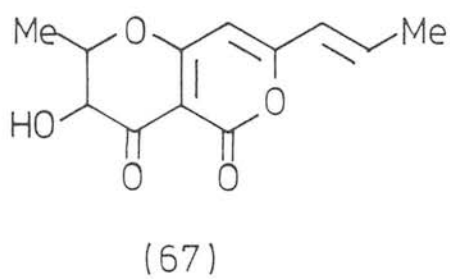
Structural analysis of LL-D253α (16), in particular the 1,3,5-oxygenation pattern of the aromatic ring, suggests a polyketide derivation but the carbon skeleton cannot be obtained by the simple folding of an unsubstituted hexaketide progenitor. Several plausible routes, which accommodate the two-carbon side-chain and which have some precedent, were envisaged.

In Scheme 12 the skeleton is formed by two successive C-methylations of a pentaketide precursor which might fold one of two ways. Both carbons of the side-chain would then originate from the C<sub>1</sub> pool. This unusual situation was observed for the 1-hydroxyethyl group of pactamycin, both carbons being labelled by [Me-<sup>13</sup>C]methionine<sup>55</sup>.

Scheme 13 depicts the addition of a C<sub>1</sub> unit to a hexaketide chain. Initial attachment could occur on any one of three chain positions. Ring closure and decarboxylation then gives the LL-D253α skeleton. The incorporation of a C<sub>1</sub> unit into a carbocyclic ring is very uncommon but a good



Scheme 16

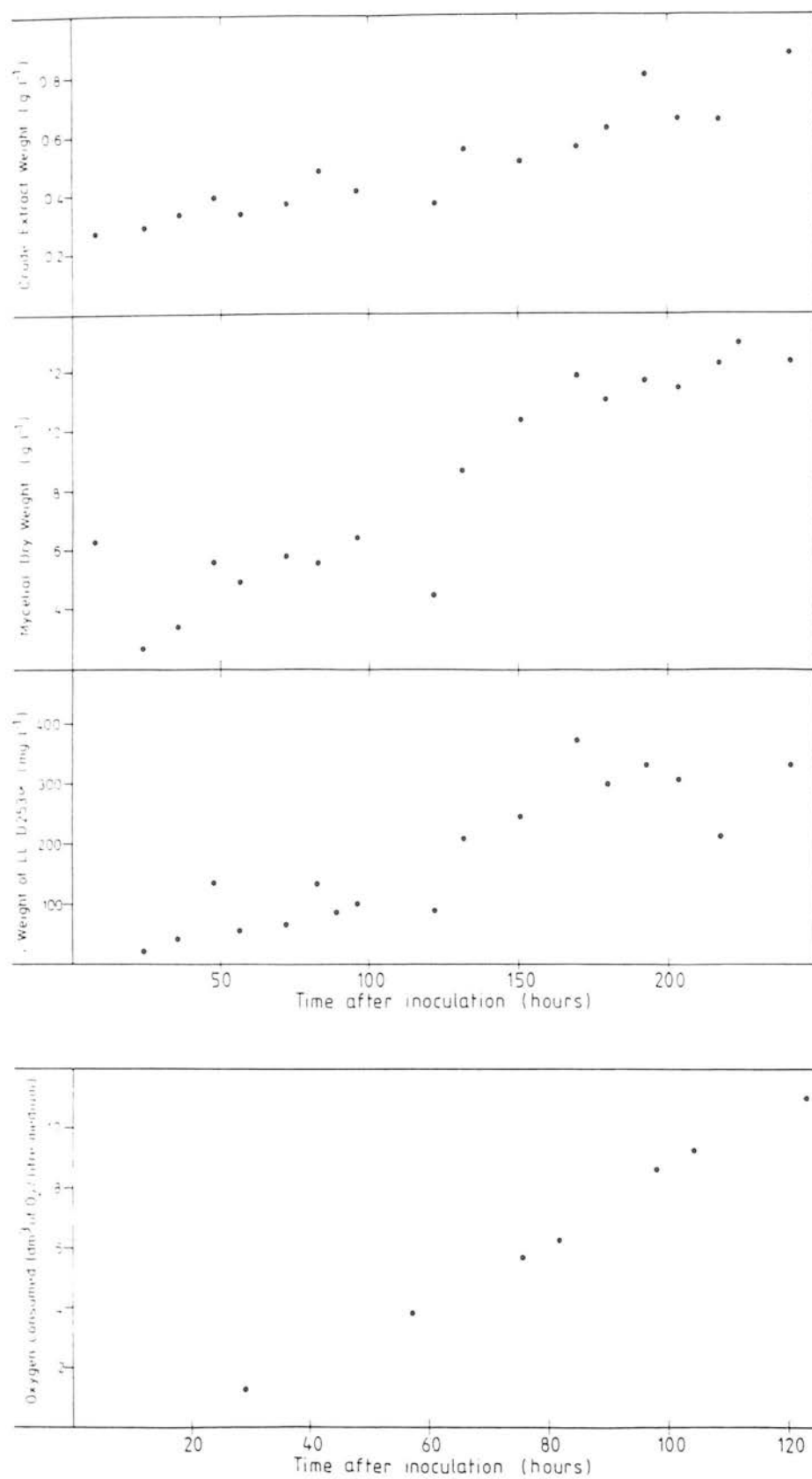


example of such a process is found in the biosynthesis of diplosporin (64). [Me-<sup>13</sup>C]methionine labelled the 2 and 5 positions as shown<sup>56</sup> (Scheme 14).

The oxidation levels of the hydroxyethyl side-chain carbons suggested that the pathway illustrated in Scheme 15 might be more likely. Here, a prenyl unit is attached to a pentaketide precursor and oxidative cleavage then gives the side-chain. Mycophenolic acid (65) is known to be formed in an analogous manner from 6-farnesyl-5,7-dihydroxy-4-methylphthalide<sup>57</sup> (66).

A pathway whereby two preformed polyketide chains are condensed, as exemplified in Scheme 16, could also account for the LL-D253 $\alpha$  skeleton. On the basis of starter effects, two-chain pathways have been proposed for radicinin<sup>58</sup> (67) and citromycetin<sup>59</sup> (68) but, other than by eliminating alternative mechanisms, it is difficult to acquire additional evidence for such proposals. Two-chain mechanisms suggested for the formation of sclerin (69) and griseofulvin (70) have subsequently proved unnecessary<sup>60,61</sup> and a single-chain pathway for citromycetin has also been suggested<sup>62</sup>.

All of these hypothetical schemes represent rather unusual variations on polyketide biosynthesis. The object, therefore, of this study was to clarify which, if any, of these routes was correct, initially at least by studying the incorporation of <sup>13</sup>C-labelled acetates.



**Figure 10** Growth and production parameters of *Phoma pigmentivora*

### 3.5 Biosynthetic Studies of LL-D253 $\alpha$

#### 3.5.1 Preliminary Investigations

*Phoma pigmentivora* was grown in shake-culture using the published<sup>1</sup> conditions. The first requirement for biosynthetic studies was some knowledge of the way in which production depended on time. To this end, an experiment was performed in which LL-D253 $\alpha$  was isolated at various times after inoculation of the flasks. Figure 10 shows crude extract weight, mycelial dry weight and weight of LL-D253 $\alpha$  as functions of time. The consumption of oxygen, measured in a later experiment, is also shown for comparison. Both growth and production appeared to be somewhat erratic. The start of "idiophase" (the period of secondary metabolite production<sup>63</sup>), often the best time for feeding potential precursors, is not readily apparent although there are signs of an increase in weight around day 4. This is probably due to the inoculation procedure. Instead of inoculating with a spore-suspension, as is more usual, the growth flasks were inoculated with a suspension of mycelium, itself previously grown from spores. This was intended to minimise the effects of the unpredictable delays between adding the spore-suspension to the culture medium and obtaining satisfactory growth. Hence the "lag" growth period, characteristic of a spore inoculation, was not seen. Despite this precaution, however, yields of metabolite in subsequent experiments showed occasional and inexplicable variations although they were usually between 200 and 400 mg l<sup>-1</sup>.



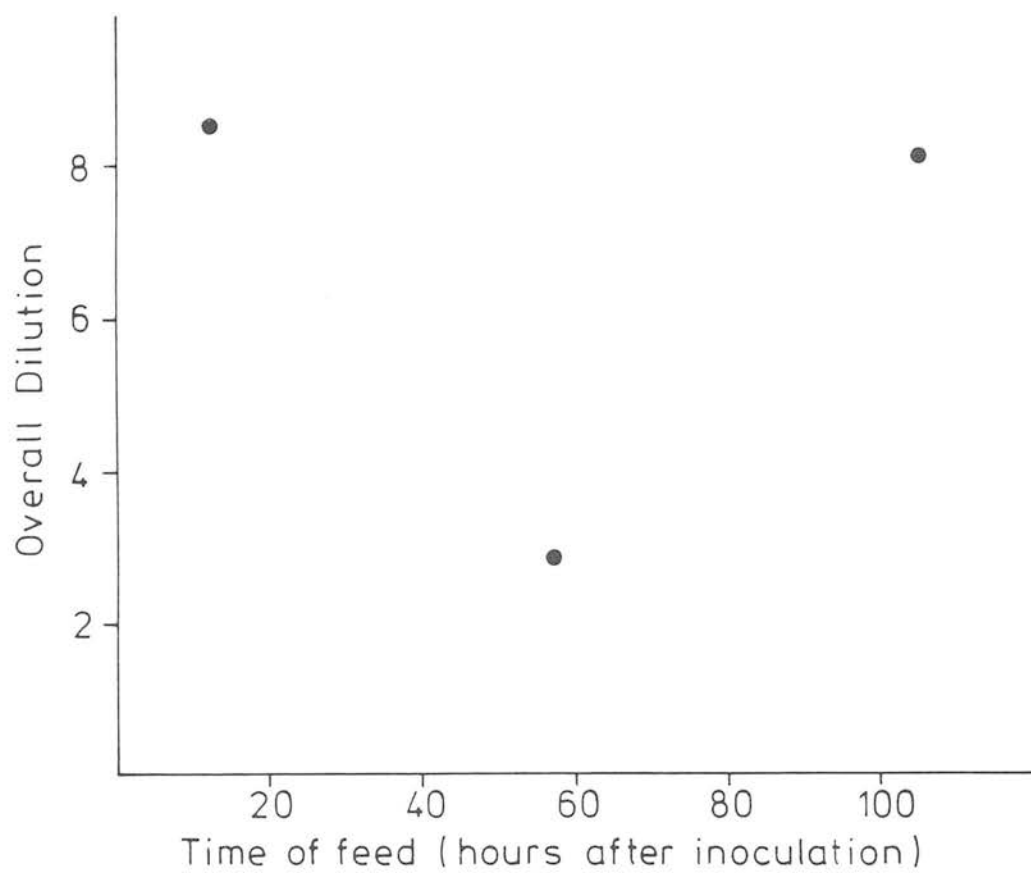


Figure 11 Dilutions of  $[2-^{14}\text{C}]$ acetates in LL-D253 $\alpha$ .

Before feeding  $^{13}\text{C}$ -labelled acetates, experiments were performed with  $[2-^{14}\text{C}]$ acetate to ascertain what levels of dilution could be expected. Samples were administered at 12 hours, 57 hours and 105 hours to separate cultures; LL-D253 $\alpha$  was isolated and purified to constant activity. Figure 11 clearly shows that the lowest dilution of 2.8 was obtained from the 57 hour feed. Assuming six acetate units per molecule gives a dilution value at each labelled site of around 17. Thus, under similar conditions, a 100%  $^{13}\text{C}$ -labelled sample of acetate would be expected to enrich each site in excess of five-fold over natural abundance. Another encouraging feature was the maintenance of good yields of LL-D253 $\alpha$  under the influence of over  $1\text{ g l}^{-1}$  of exogenous acetate.

### 3.5.2 Incorporation of $^{13}\text{C}$ -Labelled Acetates

Undiluted  $[1-^{13}\text{C}]$ -,  $[2-^{13}\text{C}]$ - and  $[1,2-^{13}\text{C}_2]$ acetates were fed to separate, 57-hour cultures of *P.pigmentivora*. The  $^{13}\text{C}$  n.m.r. spectra of the resulting samples of LL-D253 $\alpha$  indicated that a high degree of  $^{13}\text{C}$ -enrichment had taken place, in accordance with the predictions of the  $^{14}\text{C}$  experiment. However, the spectrum of the sample labelled by  $[1,2-^{13}\text{C}_2]$ acetate consisted almost entirely of complex, signals showing multiple  $^{13}\text{C}$ - $^{13}\text{C}$  couplings. Only the "terminal" carbons, *i.e.* the 2-methyl and hydroxymethylene carbons, gave the expected simple triplet, composed of a "natural abundance" centre-line and a pair of coupling satellites from an intact double-labelled acetate unit.

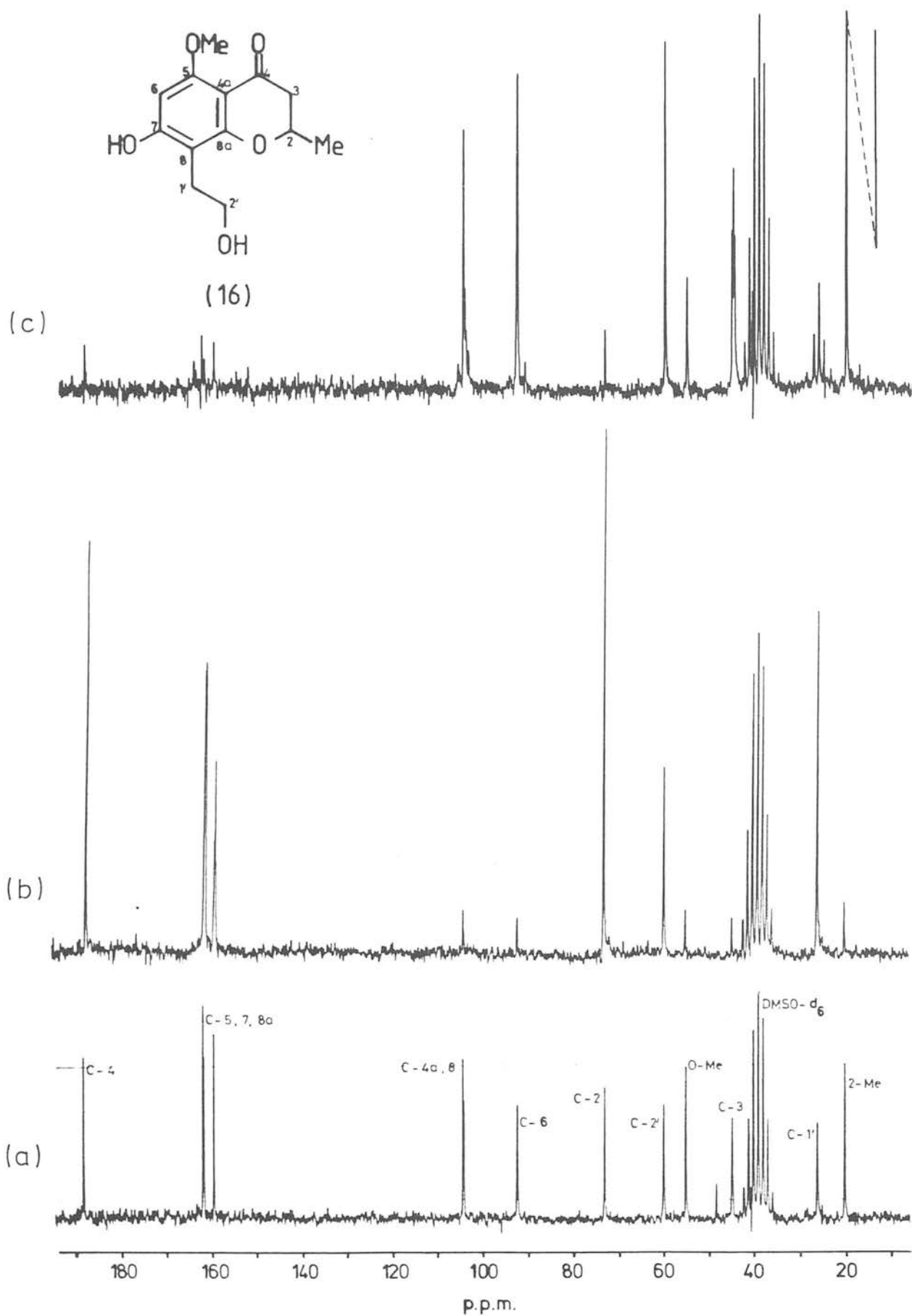
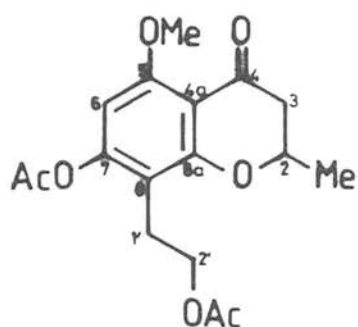


Figure 12 20 MHz  $^{13}\text{C}$  n.m.r. spectra of LL-D253 $\alpha$  (in  $\text{DMSO-d}_6$ ):  
 (a) unlabelled; (b) labelled by  $[1-^{13}\text{C}]$ acetate;  
 (c) labelled by  $[2-^{13}\text{C}]$ acetate.

Even the samples obtained from the  $[1-^{13}\text{C}]$ - and  $[2-^{13}\text{C}]$ acetate feeding experiments gave  $^{13}\text{C}$  spectra (Figure 12) which, on expansion, showed extensive long-range  $^{13}\text{C}$ - $^{13}\text{C}$  coupling. This made the signal intensities very unreliable.

Taking into account the natural abundance of  $^{13}\text{C}$  and the results of the preliminary  $^{14}\text{C}$  experiment, each labelled site should contain  $^{13}\text{C}$  to the extent of *ca.* 7% of the total sample. If each 7% is distributed evenly throughout the sample, then the probability of adjacent acetate units both carrying  $^{13}\text{C}$  label is no more than *ca.* 0.5%. That inter-acetate coupling was so extensive indicates that the addition of exogenous acetate inhibited the *in vivo* production of acetate by the fungus: LL-D253 $\alpha$  was then formed largely from the added, labelled acetate. When the acetate levels were once again low, endogenous acetate production resumed and virtually unlabelled metabolite was produced. Hence the overall level of labelling was as expected but the label was localised in relatively few molecules of LL-D253 $\alpha$ . A similar situation was observed in a study of griseofulvin<sup>61</sup>.

Although these spectra did not bear detailed interpretation, one intriguing feature was apparent (Figure 12). Both  $[1-^{13}\text{C}]$ - and  $[2-^{13}\text{C}]$ acetate labelled both of the carbons of the hydroxyethyl group, although the benzylic 1'-carbon appeared to be more enriched by  $[1-^{13}\text{C}]$ acetate than by  $[2-^{13}\text{C}]$ acetate. The reverse was the case for the 2'-carbon. Some labelling of the methoxyl group by



(17)

(c)

(b)

(a)

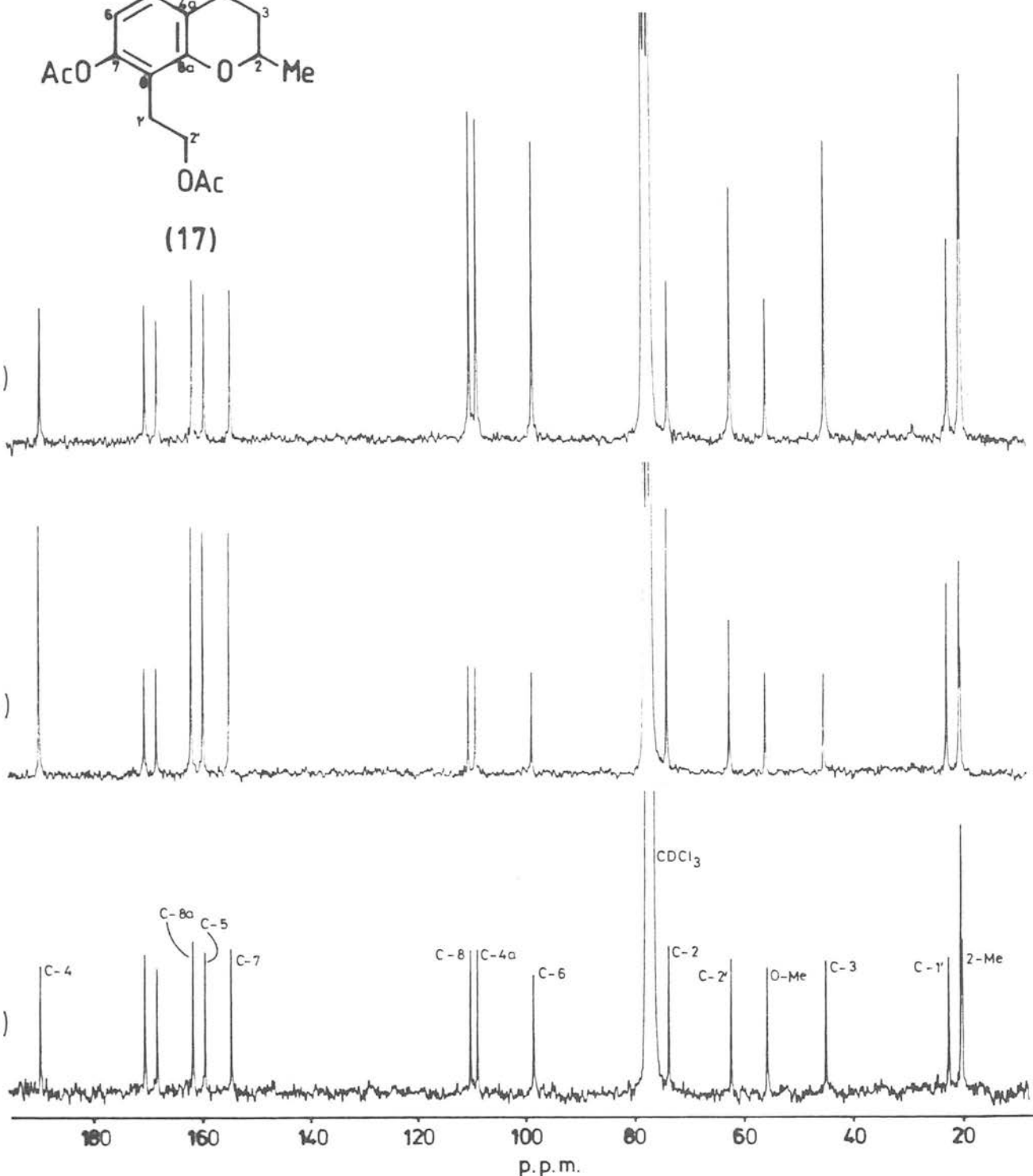


Figure 13 50 MHz  $^{13}\text{C}$  n.m.r. spectra of LL-D253 $\alpha$  diacetate (in  $\text{CDCl}_3$ ): (a) unlabelled; (b) labelled by  $[1-^{13}\text{C}]$ -acetate; (c) labelled by  $[2-^{13}\text{C}]$ -acetate. (Conditions:  $22^\circ$  pulse angle; 10,000 Hz spectral width; 8 K data points acquired; 0.41 seconds acquisition time; 2 second pulse delay; 32 K data points transformed; 0.61 Hz per point digital resolution; 5 Hz line broadening; 0.1 M equivalent of chromium tris-acetylacetonate present; decoupler gated ON during acquisition only).

[2- $^{13}\text{C}$ ]acetate was also indicated. The partial assignment of the  $^{13}\text{C}$  n.m.r. spectra in Figure 12 is based on chemical shift data and the multiplicities observed on single-frequency, off-resonance proton-decoupling (see Section 3.2, Table 1).

In order to circumvent the problem of multiple-labelling of individual molecules, the acetate-feeding experiments were repeated, this time using for each feed a 1:2 mixture of  $^{13}\text{C}$ -labelled and natural abundance acetate. Hence, in the  $^{13}\text{C}$  spectrum of the metabolite, the probability of observing coupling between adjacent acetate units where both units are obtained from exogenous acetate is reduced by approximately nine-fold. To partly compensate for the reduction in overall labelling, a 50% increase in the total dose of acetate was made.

Each sample of LL-D253 $\alpha$  thus obtained was converted to the diacetate before examination by  $^{13}\text{C}$  n.m.r. spectroscopy. The  $^{13}\text{C}$  spectral assignments for LL-D253 $\alpha$  diacetate are discussed in full in Section 3.2 and appear also in Table 4. Figure 13 shows the p.n.d.  $^{13}\text{C}$  n.m.r. spectra of the samples of LL-D253 $\alpha$  diacetate labelled by [1- $^{13}\text{C}$ ]- and [2- $^{13}\text{C}$ ]acetate compared to a natural abundance spectrum. These were all obtained under conditions, discussed in Section 2.4.1.5, designed to optimise the accuracy of the intensity measurements: small pulse angle, long pulse delay, inverse-gated proton decoupling, and in the presence of a paramagnetic relaxation agent. The

Table 4 <sup>13</sup>C Acetate enrichments of LL-D253α diacetate

Carbon	δ <sub>C</sub> (p.p.m.)	% enrichments		<sup>1</sup> J <sub>CC</sub> (Hz) <sup>a</sup>
		[1- <sup>13</sup> C]	[2- <sup>13</sup> C]	
2-methyl	20.0	0.0	1.6	40.0
C-2	73.6	1.5	0.2	40.0
C-3	44.9	-0.1	1.4	40.9
C-4	189.4	1.5	0.1	41.1
C-4a	108.8	-0.1	1.4	64.9
C-5	159.3	1.2	0.1	69.0
C-6	98.5	0.0	1.7	68.9
C-7	154.5	1.2	0.1	73.4
C-8	110.2	-0.1	1.5	72.6
C-8a	161.5	1.1	0.1	64.9
C-1'	22.7	0.8	0.5	37.6
C-2'	62.2	0.5	1.1	37.6
O-methyl	55.5	0.0	0.2	-

<sup>a</sup> From the sample enriched by [1,2-<sup>13</sup>C<sub>2</sub>]acetate

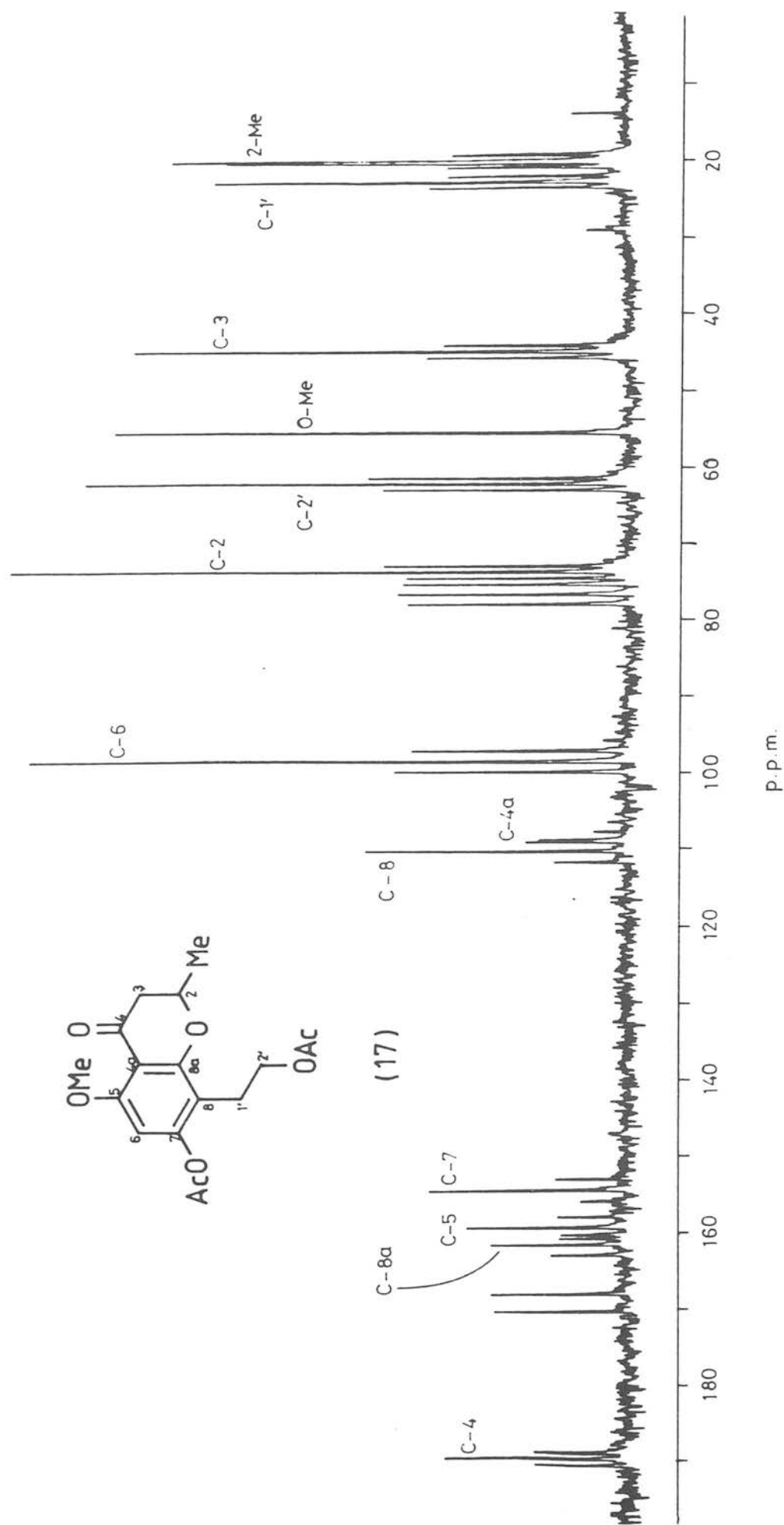


Figure 14 The 20 MHz  $^{13}\text{C}$  n.m.r. spectrum of LL-D253α diacetate (in  $\text{CDCl}_3$ ) labelled by  $[1,2-^{13}\text{C}_2]$ acetate.



enrichment figures shown in Table 4 represent the results from the averaging of the data of two such sets of spectra. Mean peak intensities from the spectra of the labelled samples were divided by the corresponding intensities of the natural abundance spectrum and the quotients were normalized to give an average of 1.00 for the acetate carbonyl signals. Percentage enrichments were then calculated using the formula given in Section 2.4.1.5.

Using this rigorous procedure for determining the enrichment data revealed that, as expected, C-2, C-4, C-5, C-7 and C-8a were all labelled by  $[1-^{13}\text{C}]$ acetate and all to about the same extent (mean enrichment of 1.3%).  $[2-^{13}\text{C}]$ Acetate similarly labelled (perhaps marginally more efficiently: mean enrichment of 1.5%) the 2-methyl carbon, C-3, C-4a, C-6 and C-8. However, as indicated by the earlier experiments, both C-1' and C-2' were labelled by both  $[1-^{13}\text{C}]$ acetate and  $[2-^{13}\text{C}]$ acetate. The combined enrichment at these two sites was, in each case, equal to the average enrichments observed at the other labelled sites but  $[1-^{13}\text{C}]$ acetate enriched the C-1' position more efficiently than it did the C-2' position.  $[2-^{13}\text{C}]$ Acetate also gave uneven labelling, but in this case heavier at C-2'. No starter effect was discernable.

Figure 14 shows the  $^{13}\text{C}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate labelled by  $[1,2-^{13}\text{C}_2]$ acetate. All the signals, except of course those due to the acetate and methoxyl groups, give characteristic triplets: coupling between acetate

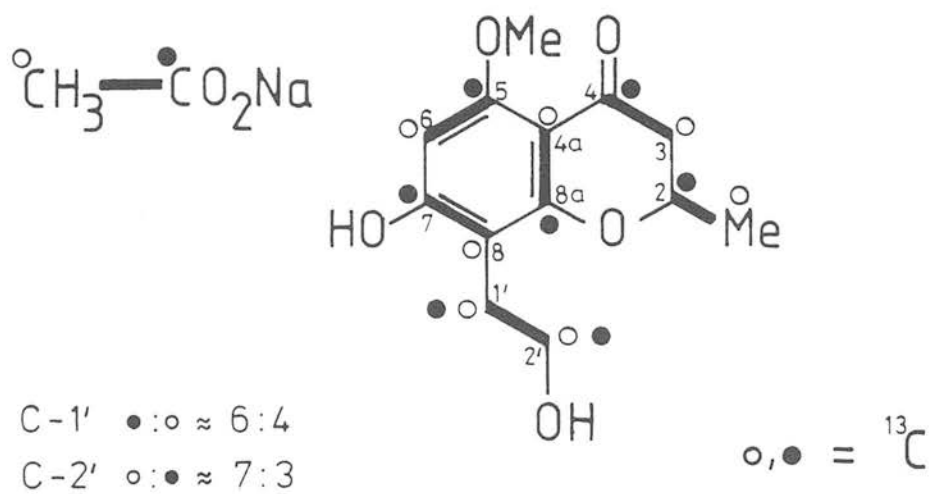
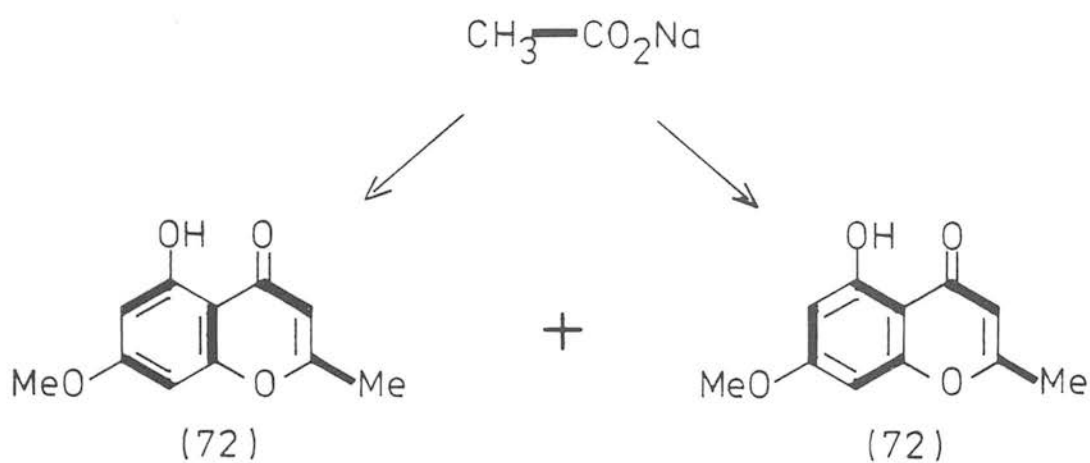
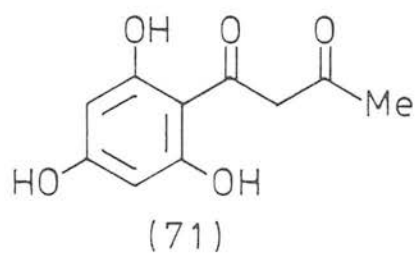


Figure 15    Labelling of LL-D253a by  $^{13}\text{C}$ -enriched acetates



Scheme 17

units appears to be absent. Table 4 includes the coupling constants obtained from the satellite lines of each triplet and shows also which pairs of carbon atoms remain contiguous during the course of biosynthesis from acetate. The value of each pair of coupling constants was sufficiently different from the others to allow unambiguous matching.

The combined results of these  $^{13}\text{C}$ -labelling experiments are illustrated in Figure 15. Only one mode of acetate incorporation into the phloroglucinol ring is observed indicating that symmetrically substituted species, *e.g.* (71), cannot be intermediate unless enzyme-bound firmly enough to prevent free rotation of the ring. This contrasts with the situation inferred in the biosynthesis of the phytoalexin, eugenin (72), where two modes of acetate incorporation were seen<sup>64</sup> (Scheme 17). A notable feature is the presence of an intact acetate bond between C-1' and C-2' despite the partial scrambling of label at these two sites revealed by the single  $^{13}\text{C}$ -labelled experiments. This observation rules out any pathway whereby the hydroxyethyl group is derived from two units of the  $\text{C}_1$  pool (as hypothesised in Scheme 12, Section 3.4). Also eliminated by these experiments is the mechanism of Scheme 13, Section 3.4, whereby C-8a was derived from the  $\text{C}_1$  pool.

### 3.5.3 Investigation of Possible Sources of the Hydroxyethyl Side-Chain

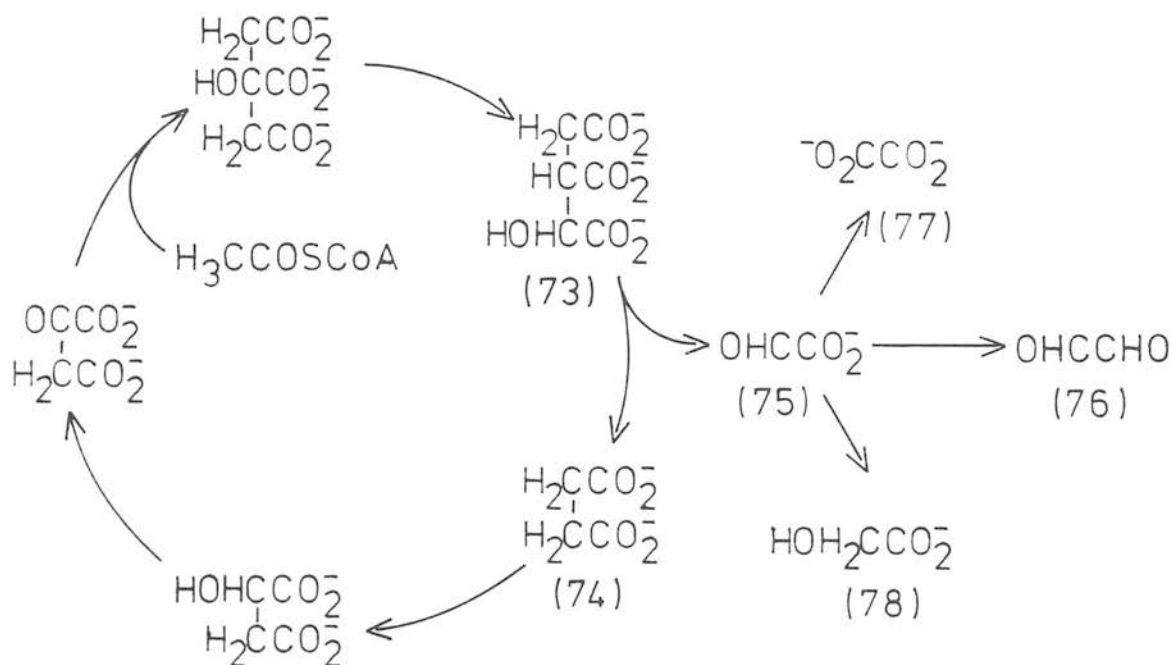
Overall and within experimental error, acetate labelled the hydroxyethyl group with the same efficiency as it did the rest of the molecule. This suggested that the hydroxyethyl group was derived from acetate in the same way, *i.e.* that the whole molecule was polyketide in nature. On the other hand, this could simply have been coincidental: a mevalonate origin for the side chain (Scheme 15, Section 3.4) would also have resulted in the incorporation of an intact acetate unit. Either way, the partial scrambling of label from C-1 and C-2 of acetate presented difficulties.

Owing to the mechanisms of the tricarboxylic acid (T.C.A.) cycle,  $[2-^{13}\text{C}]$ acetate may to some extent label carbons formally derived from C-1 of acetate<sup>61</sup> but the converse of this is not generally observed:  $[1-^{13}\text{C}]$ acetate is a more specific precursor, although due to more rapid loss as carbon dioxide it is slightly less efficient<sup>65</sup>. In any case, such a randomisation of label would not allow the retention of coupling between the side-chain carbons. The coupling satellites (Figure 14) for these carbons are no smaller than the satellites elsewhere in the spectrum.

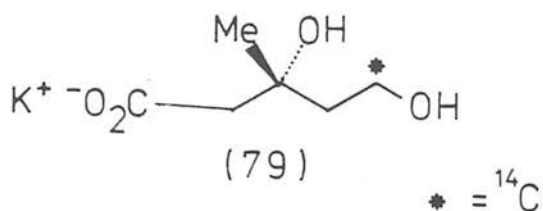
Both the randomisation and retention of coupling could be explained by the intermediacy of a symmetrical  $\text{C}_2$  unit derived from acetate. Such species could conceivably be formed *via* the glyoxylic acid cycle<sup>66</sup>, an anaplerotic pathway within the T.C.A. cycle. This pathway only occurs

Table 5    Incorporations of some possible side-chain precursors into LL-D253 $\alpha$

Tracer	Dilution	Percentage Incorporation
[1- $^{14}$ C]acetate	$2.8 \times 10^5$	7.2
[1- $^{14}$ C]glycollate	$2.0 \times 10^5$	0.14
[U- $^{14}$ C]oxalate	$1.3 \times 10^5$	4.2
[5- $^{14}$ C]mevalonate	<i>ca.</i> $1 \times 10^7$	<i>ca.</i> 0.01



Scheme 18



in micro-organisms and plants. Isocitrate (73) is divided into succinate (74) and glyoxylate (75) such that the glyoxylate could be derived from an intact acetate unit (Scheme 18). Either a reduction to glyoxal (76) or an oxidation to oxalate (77) would then give a symmetrical species which might have been the source of the hydroxyethyl group of LL-D253 $\alpha$ . The only problem with this hypothesis as it stood was that the scrambling between C-1 and C-2 of acetate was not complete. Perhaps then, the immediate precursor of the side-chain, although itself assymmetric, *e.g.* glycollate (78) or glyoxylate, was in equilibrium with a symmetrical species such as oxalate. The degree of scrambling would then reflect the rate of this equilibration relative to the rate with which the supposed precursor was incorporated into the metabolite.

In order to obtain evidence for or against this proposal, the relative incorporation of some possible side-chain precursors was examined. Sodium [1-<sup>14</sup>C]acetate, sodium[1-<sup>14</sup>C]glycollate and [U-<sup>14</sup>C]oxalic acid were administered to separate cultures of *Phoma pigmentivora*. LL-D253 $\alpha$  was isolated and purified to constant activity. In a separate experiment, the incorporation of potassium [5-<sup>14</sup>C]mevalonate (79) was also investigated. Unlike the previous experiments with <sup>14</sup>C-labelled acetate, only tracer quantities of each labelled species were used and the specific activities were correspondingly high. Table 5 shows the dilutions and percentage incorporations obtained.

As expected, acetate was incorporated most efficiently. For a fungal culture, the degree of labelling by [5-<sup>14</sup>C]-mevalonate was so small as to be negligible. Although such a negative result cannot absolutely disprove an isoprenoid origin for the hydroxyethyl group, it does make the pathway hypothesised in Scheme 15, Section 3.4, rather unlikely. The incorporation of glycollate was similarly low. Oxalate, on the other hand, labelled LL-D253α with an efficiency comparable to that of acetate.

#### 3.5.4 Incorporation of [1-<sup>13</sup>C, <sup>2</sup>H<sub>3</sub>]acetate

This last result, however, was somewhat at odds with another obtained at about the same time. Using the deuterium-induced β-isotope shifts in the <sup>13</sup>C n.m.r. spectrum of a metabolite enriched by [1-<sup>13</sup>C, <sup>2</sup>H<sub>3</sub>]acetate, it is possible to deduce not only where in the metabolite deuterium has been retained but also how many deuterium atoms are present at each site (Section 2.4.2). As exchange processes, especially for polyketides, may remove so much deuterium that the shifted signals are invisibly small, the absence of such a signal does not prove that acetate-derived hydrogen is lost by the mechanistic demands of the biosynthetic pathway. However, if shifted signals are present, then a pathway must exist which allows the retention of the corresponding number of acetate-derived hydrogens. The method can thus provide information limiting, for instance, the possible oxidation states of biosynthetic intermediates.

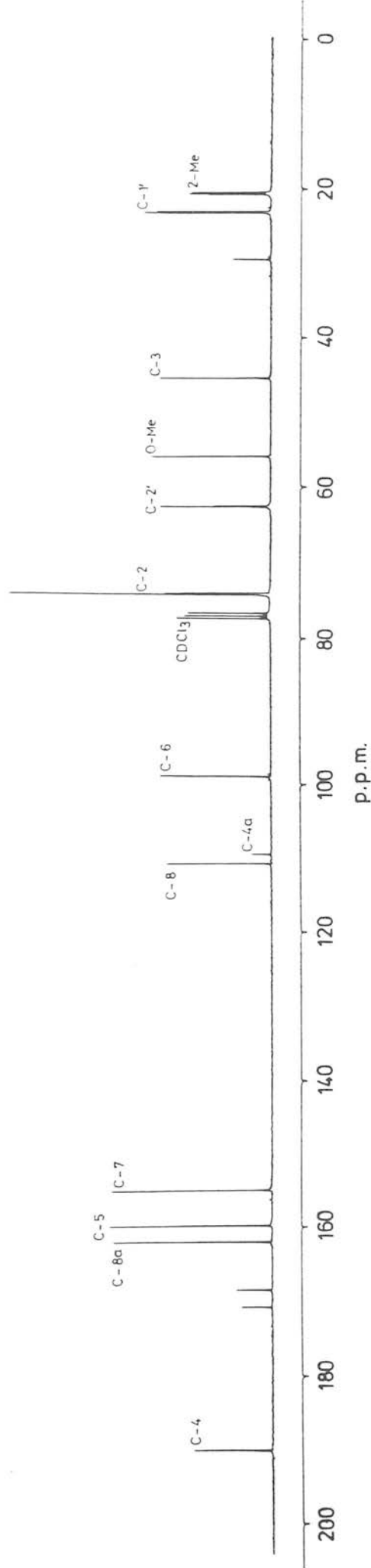
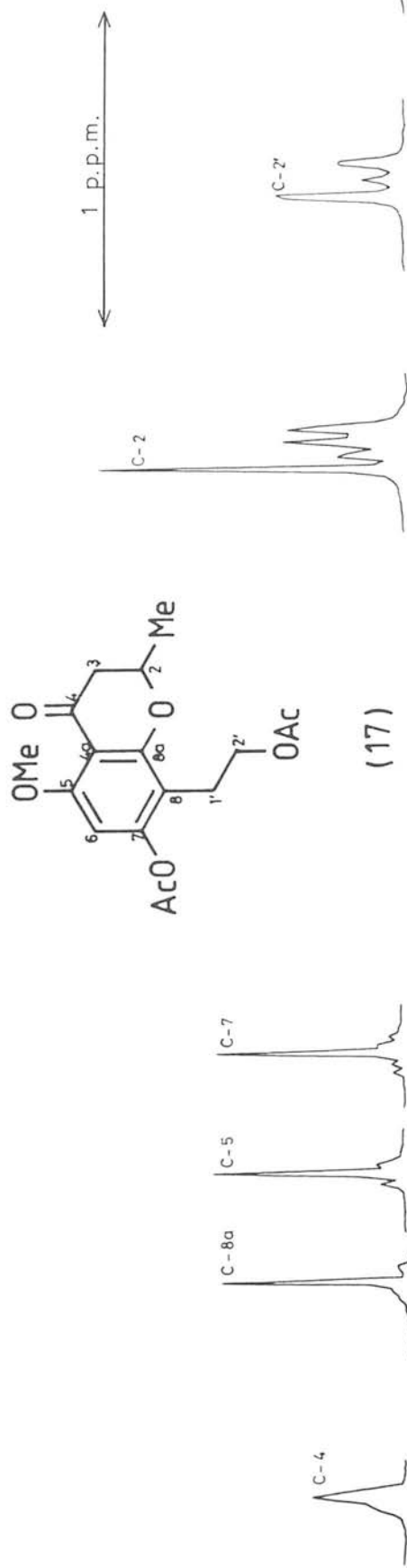


Figure 16 The 90.6 MHz  $^{13}\text{C}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate (in  $\text{CDCl}_3$ ) labelled by  $[1-^{13}\text{C}, 2\text{H}_3]$ acetate



Figure 16 shows the  $^{13}\text{C}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate isotopically enriched by the incorporation of  $[1-^{13}\text{C}, 2\text{H}_3]\text{acetate}$ . (The labelled precursor was again first diluted with two equivalents of natural abundance material). Expansions of the signals from carbons labelled by C-1 of acetate are included. The spectrum contains a number of interesting features which are discussed below. However, the signals due to C-1' and C-2' are most significant in terms of possible side-chain precursors. Each of these signals shows isotopically shifted resonances corresponding to the incorporation of up to two deuteriums at the other carbon atom. Thus oxalate, despite its efficient incorporation, cannot be an obligatory precursor of the hydroxyethyl group. Moreover, the existence is proved of a pathway to the hydroxyethyl group from acetate which requires the loss of only one acetate hydrogen.

If, as now seemed likely, oxalate played no part in the biosynthesis of the LL-D253 $\alpha$  skeleton, only one explanation for its incorporation remained. The enzymatic decarboxylation of oxalate to formate is well established<sup>67</sup>: label from oxalate could thereby enter the  $\text{C}_1$  pool and ultimately reach the methoxyl group, almost certainly derived *via* methionine. Demethylation of the 5-methoxyl group of LL-D253 $\alpha$  by boron trichloride<sup>51</sup> proceeded in *ca.* 80% yield and, when applied to the oxalate-labelled sample, resulted in the loss of 98% of the total activity. Incorporation of oxalate into the rest of the molecule was therefore negligible.

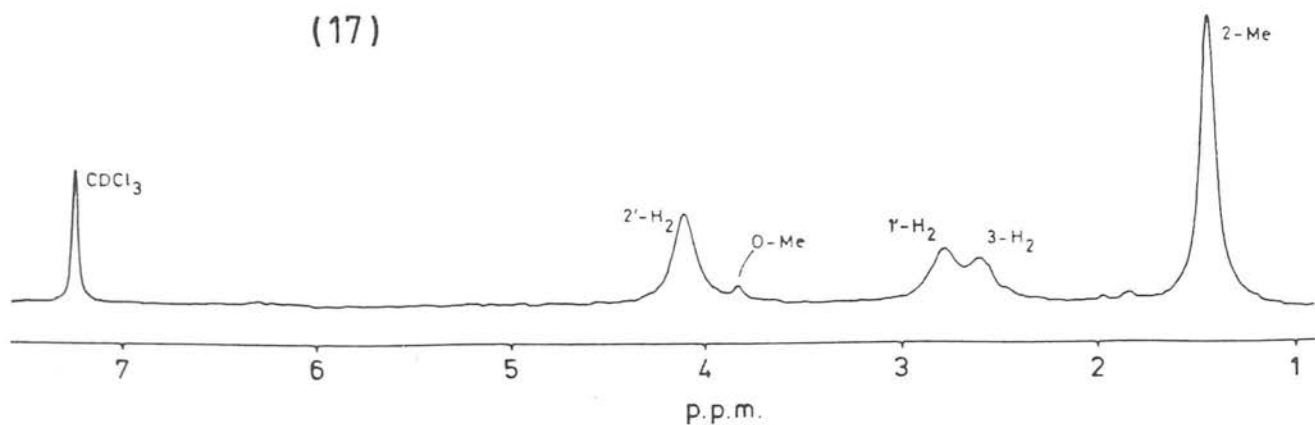
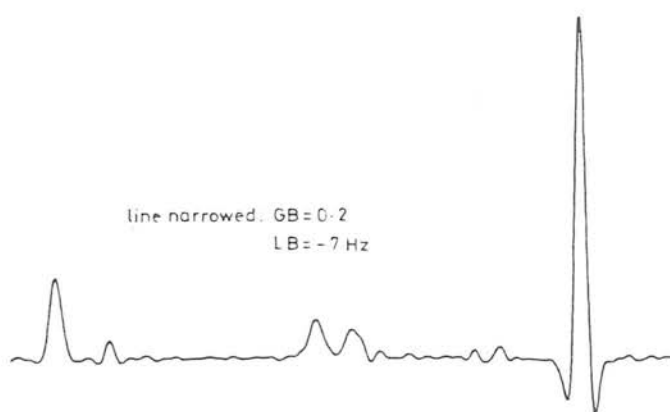
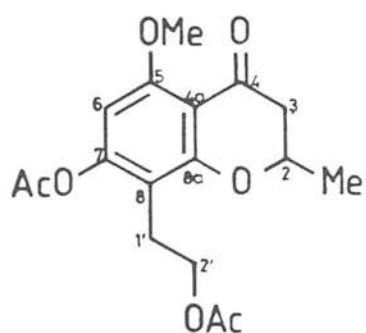
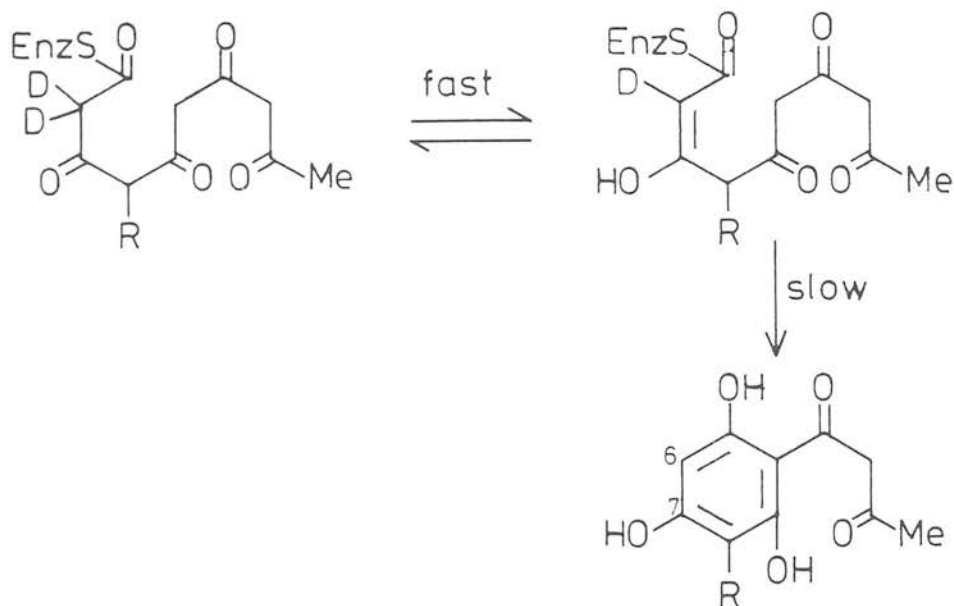


Figure 17 The 55.3 MHz <sup>2</sup>H n.m.r. spectrum of LL-D253α diacetate (in CHCl<sub>3</sub>) labelled by [1-<sup>13</sup>C, <sup>2</sup>H<sub>3</sub>]acetate

Apart from C-1' and C-2', only two other carbons showed isotopically shifted signals (Figure 16) due to the incorporation of deuterium from  $[1-^{13}\text{C}, ^2\text{H}_3]\text{acetate}$ . The C-2 resonance shows three shifted signals corresponding to the retention of one, two and three deuterium atoms in the 2-methyl group. This indicates that C-2 and the 2-methyl group are derived from an acetate starter unit as might be expected. C-4 gives a rather poorly resolved signal, but a single shoulder is visible downfield of the main peak suggesting one deuterium only at C-3. Although heavy isotope substitution almost always gives upfield shifts, a downfield  $\beta$ -shift of a carbonyl signal has been previously reported<sup>68</sup>. Carbons 8a and 7, of course, do not give any isotopically shifted signal, but, less predictably, neither does C-5. Only a small pair of coupling satellites are visible and these are ascribed to  $^2J_{\text{CC}}$  coupling in the small proportion of molecules carrying more than one labelled acetate unit in the phloroglucinol ring. The  $^2\text{H}$  n.m.r. spectrum (Figure 17) of this sample confirmed the absence of deuterium at C-6.

This last observation may reflect the retention of the 7-hydroxyl group in the biosynthesis of LL-D253 $\alpha$ . It has been suggested<sup>69</sup> that the enzyme-controlled configuration of a polyketide chain, required for cyclisation to occur, can be achieved by the formation of double bonds. Enolisation would allow this to occur without necessitating the loss of ketide oxygen but rapid interconversion with the keto-



Scheme 19

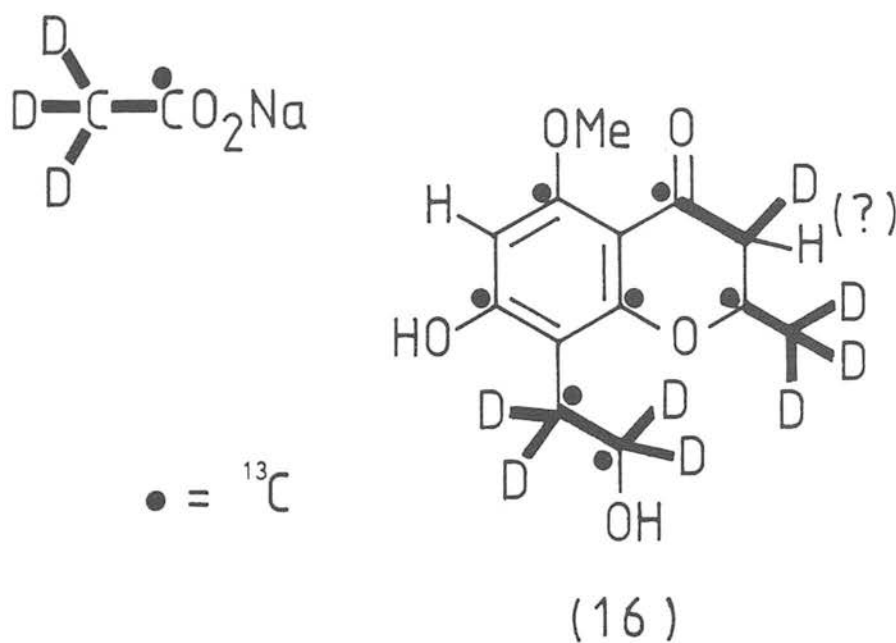


Figure 18 Labelling of LL-D253 $\alpha$  by  $[1-^{13}\text{C}, ^2\text{H}_3]\text{acetate}$

form would result in extensive exchange of acetate-derived hydrogen before cyclisation and aromatisation occurred (Scheme 19). Alternatively, the double bond could be generated by reduction of the appropriate carbonyl group followed by dehydration. As this would not be readily reversible, loss of acetate-derived hydrogen would be limited. In accordance with these ideas, it was found<sup>69</sup> that metabolites which retained the relevant hydroxyl group in the final structure showed relatively lower retention of the corresponding acetate-derived hydrogen than did deoxygenated metabolites. In the case of LL-D253 $\alpha$ , the 7-hydroxyl is retained (see below) so the lack of deuterium at C-6 of this sample is consistent with these earlier observations.

In summary, deuterium from [1-<sup>13</sup>C, <sup>2</sup>H<sub>3</sub>]acetate labelled LL-D253 $\alpha$  as shown in Figure 18. These results are clear-cut except possibly for the number of deuteriums retained at C-3. Only one shoulder could be seen on the C-4 resonance but carbonyl carbons do not make very suitable reporter nuclei for the  $\beta$ -shift method of detecting deuterium. The incorporation of [2-<sup>13</sup>C, <sup>2</sup>H<sub>3</sub>]acetate (alpha-shift technique; Section 2.4.2) might give clearer results in this case.

### 3.5.5 Mechanism of Pyranone Ring-Formation - Incorporation of [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate

The number of acetate hydrogens which can be retained at C-3 is relevant to possible mechanisms of formation of the pyranone ring. Additional information on this point came from

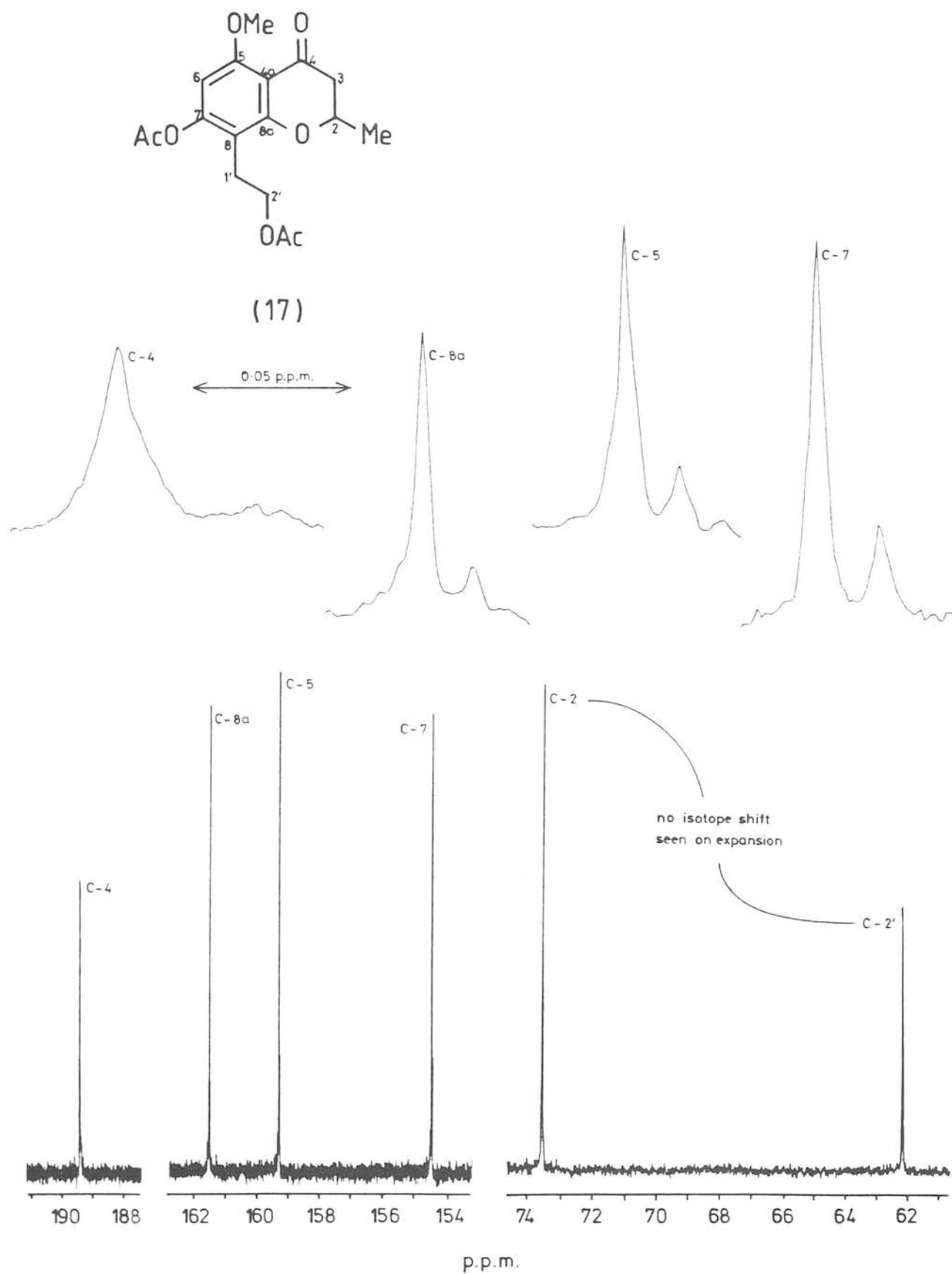


Figure 19 Part of the 100.6 MHz  $^{13}\text{C}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate (in  $\text{CDCl}_3$ ) labelled by  $[1-^{13}\text{C}, ^{18}\text{O}_2]\text{acetate}$

Table 6  $^{18}\text{O}$ -induced isotope shifts in the  $^{13}\text{C}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate

Carbon	$\delta^a$	$\Delta\delta^b$	$^{16}\text{O} : ^{18}\text{O}^c$
C-4	189.4	4.4 <sup>e</sup>	94 : 6
C-8a	161.5	1.6 <sup>e</sup>	83 : 17
C-5	159.3	1.7 <sup>e</sup>	83 : 17
C-7	154.5	2.1 <sup>e</sup>	81 : 19
C-2'	62.2	2.9 <sup>f</sup>	85 : 15 <sup>d</sup>

<sup>a</sup> In p.p.m. downfield from TMS in 100.6 MHz spectrum ( $\text{CDCl}_3$ )

<sup>b</sup> In p.p.m./100, upfield from unshifted signal

<sup>c</sup> Integrated intensity ratio of unshifted and shifted signals

<sup>d</sup> Obtained from Dupont 210 curve analyser

<sup>e</sup> From incorporation of  $[1-^{13}\text{C}, ^{18}\text{O}_2]\text{acetate}$

<sup>f</sup> From incorporation of  $^{18}\text{O}_2$

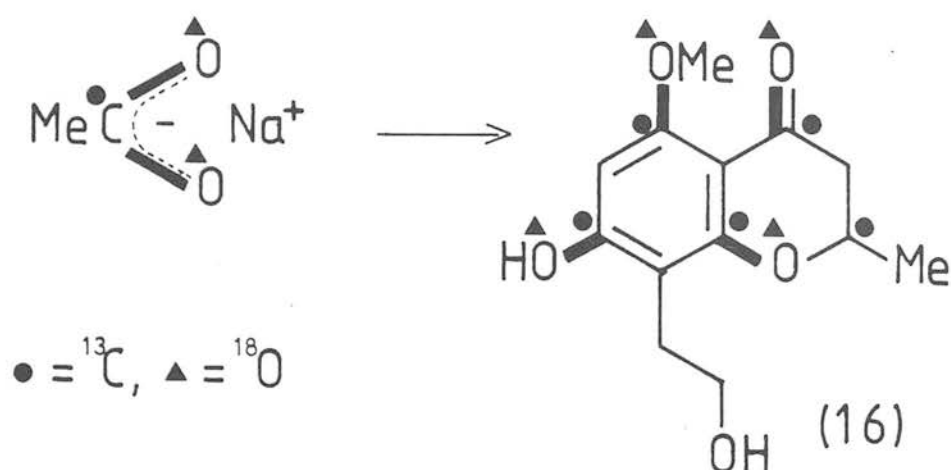


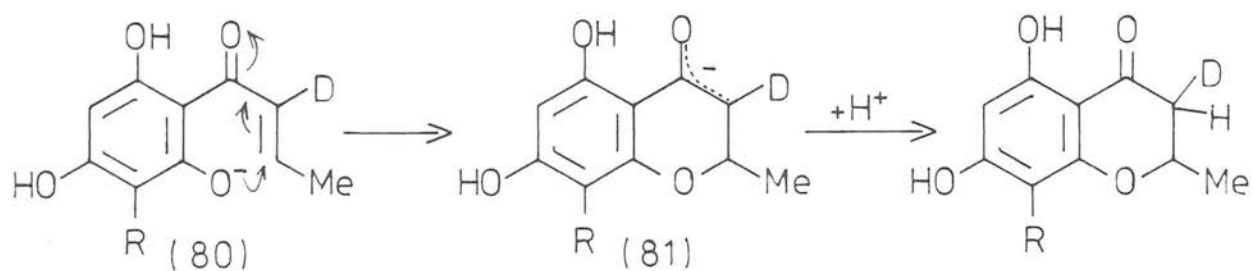
Figure 20 Labelling of LL-D253 $\alpha$  by  $[1-^{13}\text{C}, ^{18}\text{O}_2]\text{acetate}$

the results of incorporating  $[1-^{13}\text{C}, ^{18}\text{O}_2]\text{acetate}$  into LL-D253 $\alpha$ . This labelling technique (Section 2.4.3) allows the detection of intact  $^{13}\text{C}-^{18}\text{O}$  bonds by the presence of  $^{18}\text{O}$ -induced isotope shifts in the  $^{13}\text{C}$  n.m.r. spectrum of an enriched metabolite.

Figure 19 shows the  $^{13}\text{C}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate thus enriched. Resonances due to oxygen bearing carbons are shown in expanded form. Isotopically shifted signals are evident for C-5, C-7 and C-8a indicating that the corresponding carbon-oxygen bonds remained intact during the course of biosynthesis. The C-4 resonance, however, shows only a very small shifted signal. Chemical exchange *via* the hydrated form of the carbonyl group is probably responsible for the much greater loss of  $^{18}\text{O}$  seen at this position. No other signals showed any sign of an isotopic shift. To ensure that the shifted signals were not in fact due to long-range  $^{13}\text{C}-^{13}\text{C}$  coupling, the sample was re-examined using a pulse sequence<sup>70,71</sup> designed to eliminate such coupling: all the shifted signals were still present.

Table 6 summarises the isotope-shifts seen in this experiment (and that resulting from a fermentation under  $^{18}\text{O}_2$  - see below) and Figure 20 shows the incorporation of intact carbon-oxygen bonds from acetate. If, as the  $\beta$ -shift experiment suggested, there is only one acetate hydrogen retained at C-3, the incorporation of an intact, acetate-derived, carbon-oxygen bond between the pyranone ring oxygen and C-8a, rather than C-2, indicates a ring-closure mechanism similar to that of Scheme 20. This shows





Scheme 20 Possible mechanism of pyranone ring-closure

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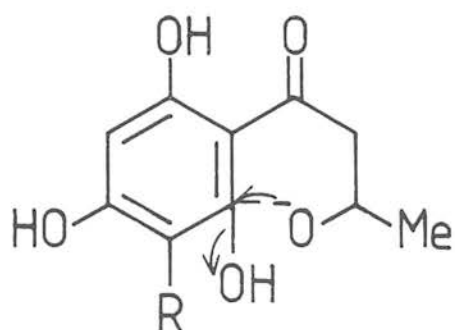
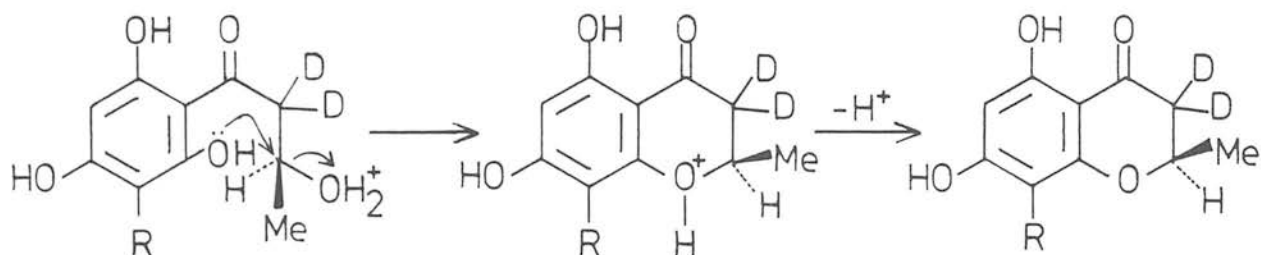


Figure 21 Precluded mechanism for pyranone ring-closure

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Scheme 21 Alternative pyranone ring-closure if two deuteriums can be retained at C-3



conjugate addition of the phenolic hydroxy group of the acrylophenone intermediate (80) to the  $\alpha,\beta$  unsaturated ketone function. A ring-closure in the opposite sense (Figure 21) is excluded. As the natural product has the  $2R$  configuration<sup>1,3</sup>, the ring-closure must proceed under enzymatic control. If the mechanism of Scheme 20 is correct, protonation of the intermediate enolate species (81) might therefore also occur stereospecifically. This appears to be the case in the corresponding chalcone to flavanone ring-closure where addition occurs with *cis* geometry to give the  $2S$  product<sup>72</sup>. It seemed worth pursuing this point as, if it could be established that acetate hydrogen was retained stereospecifically at C-3, this would obviously confirm that only one such hydrogen was present.

The axial and equatorial hydrogens at C-3 have almost coincident chemical shifts and even at high field strengths form part of a complex ABX system. Figure 22 shows the  $^1H$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate and the effects of adding increasing quantities of a lanthanide shift reagent,  $Eu(fod)_3$  (fod = 1,1,1,2,2,3,3-heptafluoro-7,7-dimethyloctane-4,6-dionate). At 0.3 molar equivalents of  $Eu(fod)_3$ , the two C-3 hydrogen resonances are well resolved, appearing at 3.58 p.p.m. (dd,  $J = 16.5, 2.5$  Hz) and 3.94 p.p.m. (dd,  $J = 16.5, 13$  Hz). Drieding models reveal that the large difference in the two vicinal coupling constants is only consistent with a conformation whereby the 2-methyl group occupies the equatorial position. As the  $3\beta$  hydrogen

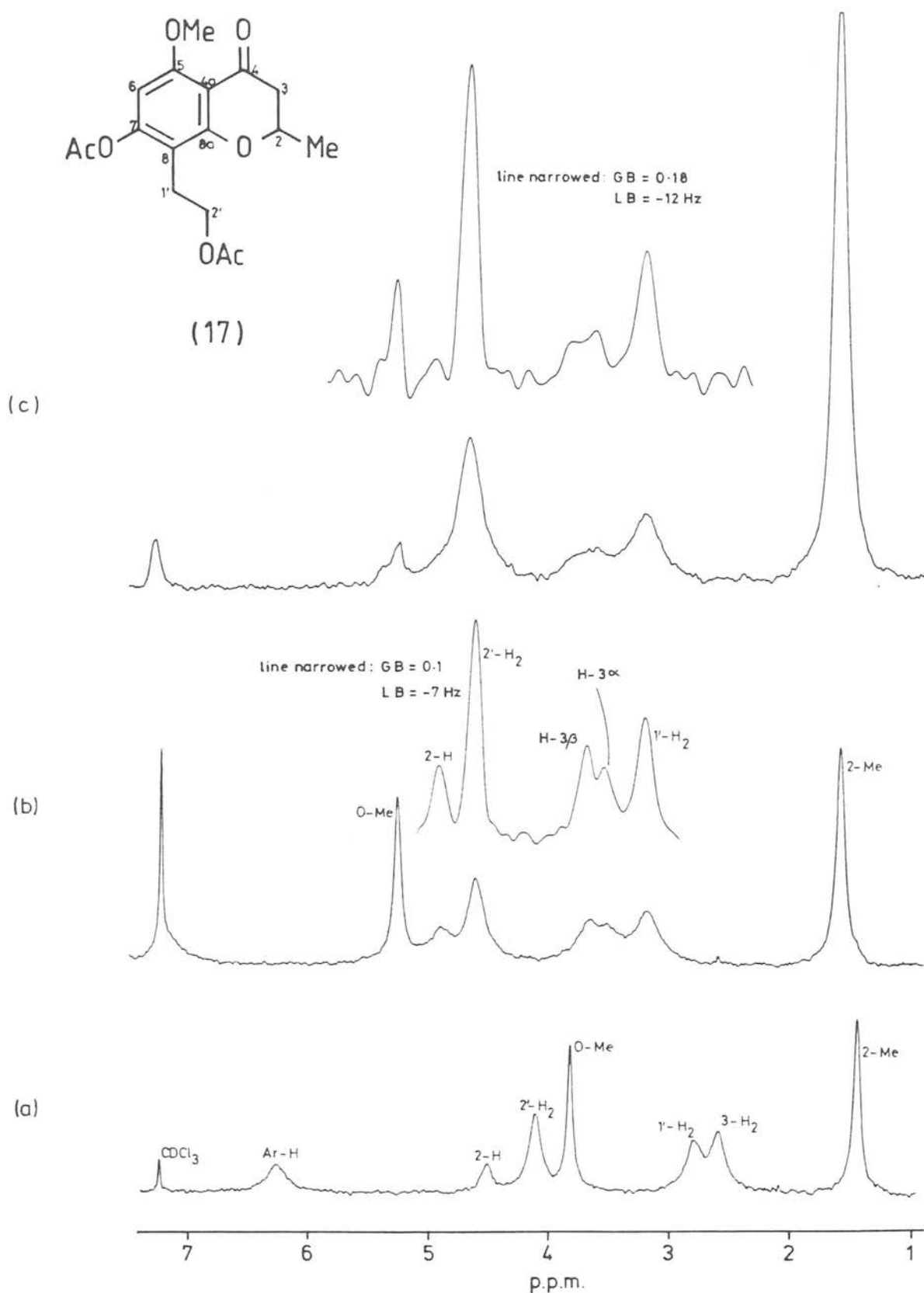


Figure 23 55.3 MHz  $^2\text{H}$  n.m.r. spectra of LL-D253 $\alpha$  diacetate (in  $\text{CHCl}_3$ ): (a) labelled by  $\text{D}_2\text{O}$ ; (b) labelled by  $\text{D}_2\text{O}$  and in the presence of 30 mol %  $\text{Eu}(\text{fod})_3$ ; (c) labelled by  $[\text{D}_3]\text{acetate}$  and in the presence of 30 mol %  $\text{Eu}(\text{fod})_3$

possesses a *trans*-diaxial relationship to the 2 $\alpha$  hydrogen, it is assigned to the signal at 3.94 p.p.m. which shows a vicinal coupling of 13 Hz. The 60° dihedral angle between the 2 $\alpha$  and 3 $\alpha$  hydrogen-carbon bonds independently assigns the 3 $\alpha$  hydrogen to the signal at 3.58 p.p.m. with its 2.5 Hz coupling.

A generally deuterium-enriched sample of LL-D253 $\alpha$  was prepared by the simple expedient<sup>73</sup> of growing *P. pigmentivora* in a medium in which 5% of the water had been replaced by D<sub>2</sub>O. The <sup>2</sup>H n.m.r. spectrum of the diacetate (Figure 23), also obtained after the addition of 0.3 molar equivalents of Eu(fod)<sub>3</sub>, showed that the two C-3 hydrogen resonances could also be resolved in the deuterium spectrum. Finally, repeating the experiment with a sample of LL-D253 $\alpha$  diacetate isotopically enriched by the incorporation of [<sup>2</sup>H<sub>3</sub>]acetate (Figure 23) indicated that both the 3 $\alpha$  and 3 $\beta$  positions were labelled by deuterium.

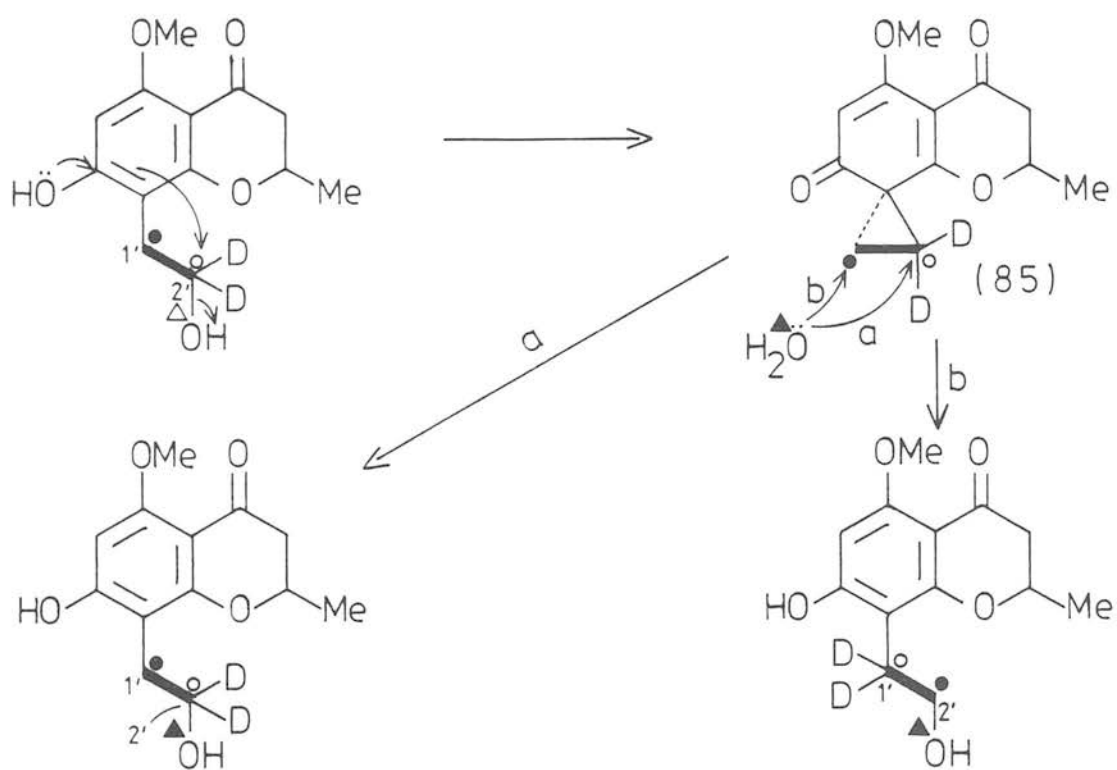
This result suggests two interpretations. If the mechanism of Scheme 20 is correct, then protonation of the intermediate enolate (81) must occur non-stereospecifically. Alternatively, the  $\beta$ -shift experiment could be misleading and the biosynthetic pathway may in fact allow the retention of up to two acetate-derived hydrogens at C-3. A ring-closure mechanism such as shown in Scheme 21 would then be permissible. At present, no further information pertinent to this problem is available.



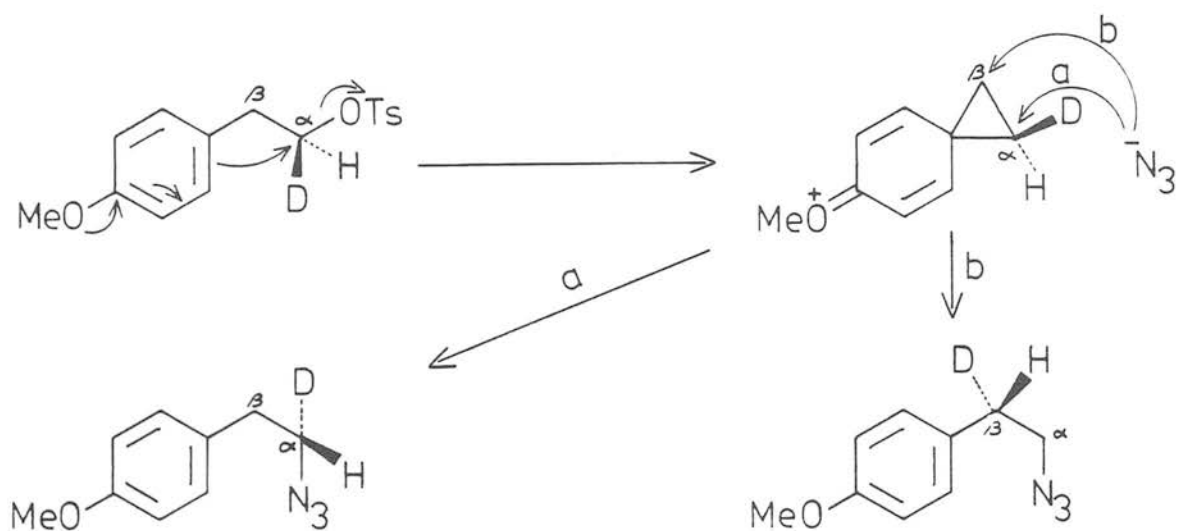
3.5.6 Mechanism of Skeletal Formation -  
Incorporation of  $^{18}\text{O}_2$  Gas

In readdressing the original problem, *i.e.* by what means are the twelve carbon atoms of the LL-D253 $\alpha$  skeleton assembled, several pieces of evidence require consideration. Firstly, the skeleton appears to be entirely polyketide. Supporting this conclusion are: the equal levels of labelling throughout the carbon framework by single-labelled  $^{13}\text{C}$  acetates, the retention of six intact carbon-carbon bonds from [1,2- $^{13}\text{C}_2$ ]acetate and the failure of [5- $^{14}\text{C}$ ]mevalonate to significantly label the metabolite. Secondly, the results of incorporating [1,2- $^{13}\text{C}_2$ ]acetate indicate that intermediates containing a symmetrically substituted phloroglucinol ring are unlikely. Thirdly, the part randomisation of label from acetate between C-1' and C-2' indicates that these carbons may become equivalent during the course of biosynthesis. Fourthly, the results of the  $\beta$ -shift experiment preclude intermediates in which the carbons of the hydroxyethyl unit originally from C-2 of acetate possess less than two acetate-derived hydrogens.

To accommodate these observations, the pathway shown in Scheme 22 is proposed. Two preformed polyketide chains are condensed to give, after aromatisation and pyranone ring-formation, the 8-acetyl-4-chromanone (82). No information on the actual timing of these steps is available; the Scheme shows two  $\text{C}_6$  chains for which condensation



Scheme 23 Possible mechanism of  $^{13}\text{C}$  scrambling



Scheme 24



would have to precede aromatisation. Other combinations,  $C_2$  plus  $C_{10}$  or  $C_4$  plus  $C_8$  (Figure 24) are also possible; here aromatisation could occur before addition of the smaller chain but the first-formed aromatic products would have to be firmly enzyme-bound to avoid randomisation of carbon-label in the symmetrical aromatic ring.

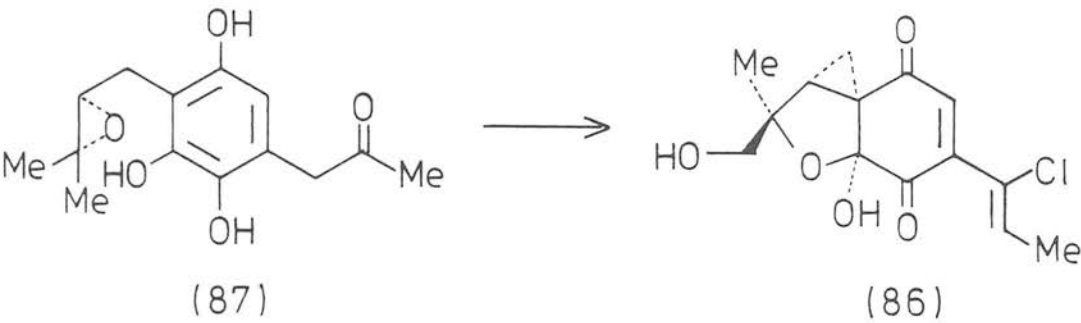
Reduction and dehydration of the 8-acetyl intermediate (82) (*cf.* fatty acid biosynthesis) would give the vinyl species (83). A mono-oxygenase mediated introduction of oxygen giving the epoxide (84), followed by reductive opening in the direction shown, would then provide LL-D253 $\alpha$  (16) in which the oxygen of the hydroxyethyl group was entirely atmospherically derived. The partial randomisation of carbon label in this group can be rationalised by a subsequent process (Scheme 23), whereby the phloroglucinol ring participates in displacing the terminal hydroxyl at C-2' to give a spiro-cyclopropyl intermediate (85). Hydrolytic opening of this unstable species could occur at either methylene group, in one case (a) retaining the original labelling pattern and in the other case (b) reversing it. LL-D253 $\alpha$  which had undergone these reactions would therefore possess water-derived oxygen at C-2'.

A similar process has been implicated<sup>74</sup> in the observed randomisation of deuterium label from C- $\alpha$  to C- $\beta$  during the synthesis of some chirally labelled tyramines (Scheme 24). Aryl participation was suppressed by using the electron withdrawing methanesulphonyl group in place of the

Table 7      Significant parent-ions of LL-D253a produced  
under an  $^{18}\text{O}_2$  atmosphere

Formula	m/z		Line Heights (mm)	
	Calculated	Found	Observed	Corrected <sup>a</sup>
$^{12}\text{C}_{13}\text{H}_{16}\text{}^{16}\text{O}_5$	252.0998	252.0988	72	83
$^{12}\text{C}_{12}\text{}^{13}\text{C}_1\text{H}_{16}\text{}^{16}\text{O}_5$	253.1031	253.1028	12	(2)
$^{12}\text{C}_{13}\text{H}_{16}\text{}^{16}\text{O}_4\text{}^{18}\text{O}_1$	254.1040	254.1032	14	15

<sup>a</sup> Correction based on M:M+1:M+2 = 86.6:12.5:0.8 for a C<sub>13</sub> compound



methyl group as protection for the phenol function. Also of interest in this connection is the fungal metabolite, mikrolin (86), which contains a spiro-cyclopropyl ring attached to a cyclohexadienone ring. A biosynthetic pathway involving the epoxide (87) was suggested and supported by the incorporation of  $^{13}\text{C}$ -labelled acetates<sup>75</sup>.

The fact that randomisation of the hydroxyethyl carbons is not complete suggests two possibilities. If the process depicted in Scheme 23 occurs rapidly enough for each molecule of LL-D253 $\alpha$  to have been through several cycles, then both formation and hydrolytic cleavage of the cyclopropane ring must be highly stereospecific, resulting each time in a net retention of the original labelling. Alternatively, and probably more likely, the process is relatively slow and not very stereospecific: in this case the incomplete randomisation arises from the proportion of molecules which have not yet taken part.

Support for this latter proposal and for Scheme 23 as a whole was provided by a fermentation of *P. pigmentivora* under an atmosphere of  $^{18}\text{O}_2$  gas (using the "constant-pressure" apparatus - Appendix ). The mass-spectrum of the LL-D253 $\alpha$  showed an M+2 peak which mass-matched for  $^{12}\text{C}_{13}\text{H}_{16}^{16}\text{O}_4^{18}\text{O}$ . After allowing for the effect of the natural abundance of  $^{13}\text{C}$ , this peak was seen to comprise about 15% of the total molecular ion currents for M, M+1 and M+2 (Table 7). The sample was converted to its diacetate and this was examined by  $^{13}\text{C}$  n.m.r. spectroscopy. Of the oxygenated carbons,

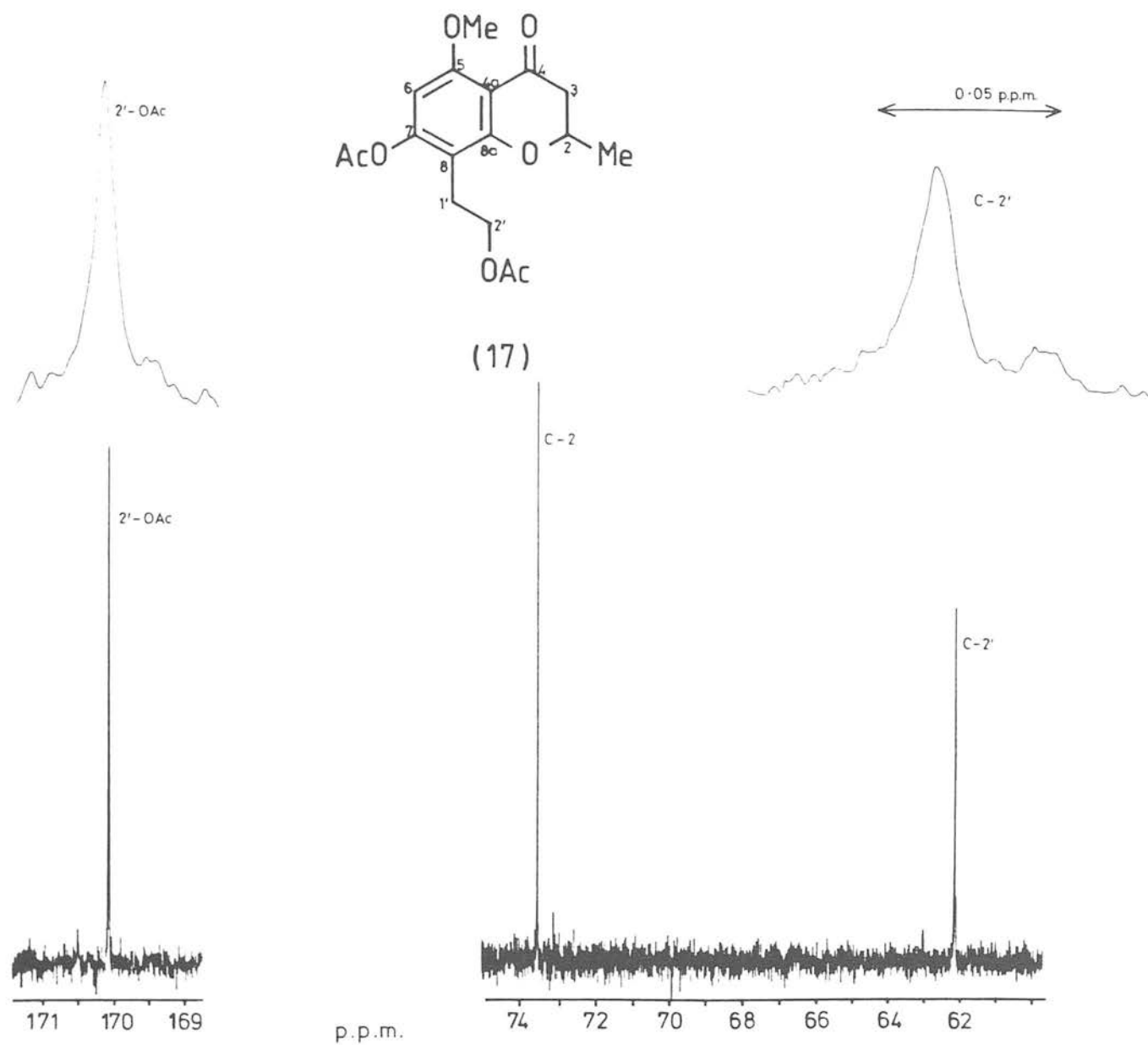


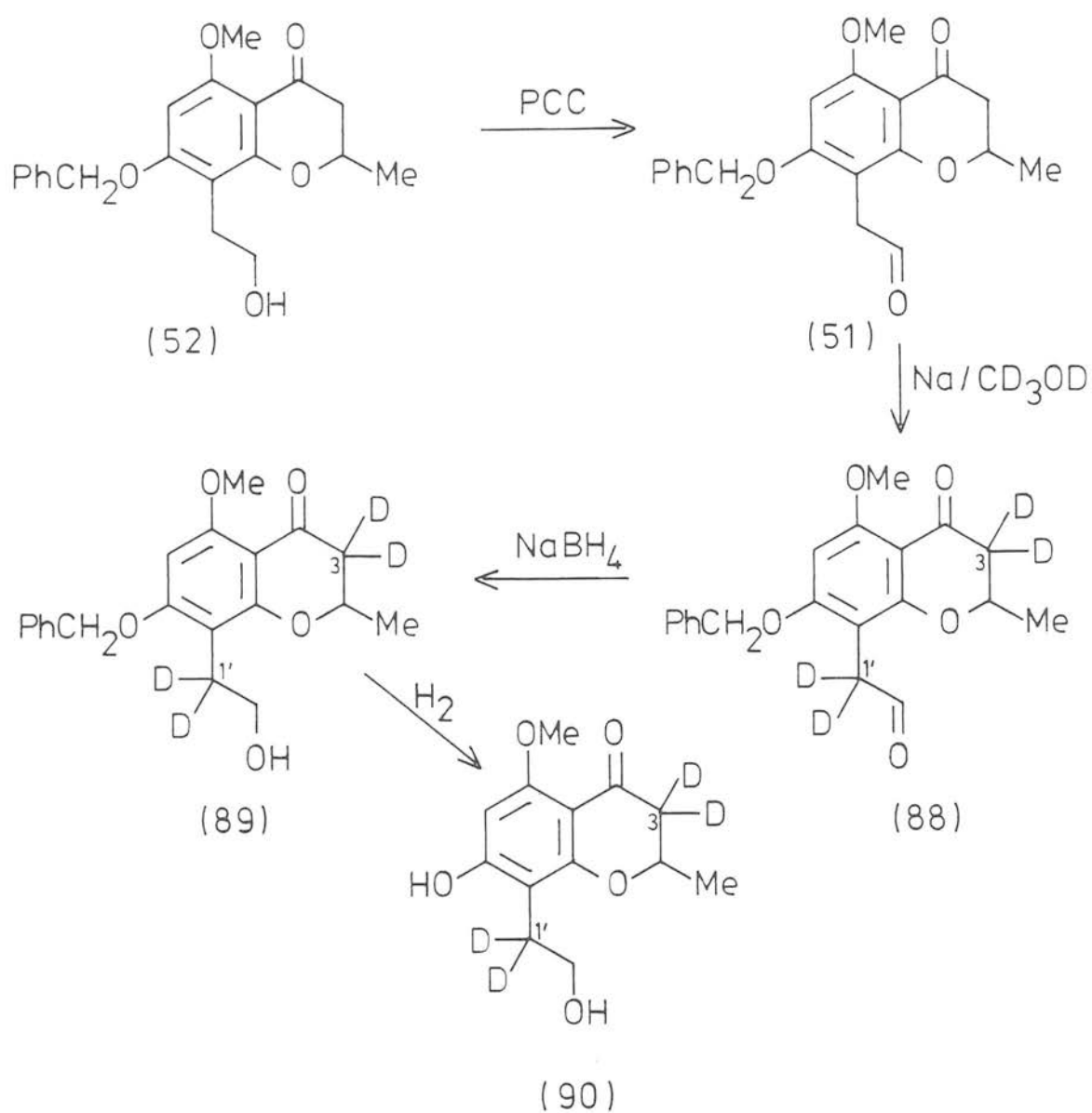
Figure 25 Part of the 100.6 MHz  $^{13}\text{C}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate (in  $\text{CDCl}_3$ ) labelled by  $^{18}\text{O}_2$  gas

C-2' was the only one to give a resonance showing any sign of an  $^{18}\text{O}$  isotope-shift (Figure 25, Table 6)\*. A curve-analyser gave the ratio of shifted to unshifted signals as 85:15. Thus, within experimental error, all of the  $^{18}\text{O}$  label was located at C-2'.

This result implies that 15% of the LL-D253 $\alpha$  had not undergone cyclopropyl ring-formation and subsequent hydrolysis. As there is no reason to believe that the extent of the randomisation process is exactly the same in every fermentation, the figure bears comparison with the observed degree of carbon randomisation in the experiments with singly  $^{13}\text{C}$ -labelled acetates. These gave an average figure of about 65% retention and 35% inversion of the original labelling of the putative acetyl intermediate (82). If formation and hydrolysis of the cyclopropyl species (85) occurs with equal facility from either direction, then in this case 30% of the metabolite would have been labelled from  $^{18}\text{O}_2$  gas.

At present, it is not clear whether the scrambling is an *in vivo* or *in vitro* process. In a brief attempt to demonstrate the latter, a specifically deuterated sample of LL-D253 $\alpha$  has been prepared (Scheme 25). LL-D253 $\alpha$ -benzyl ether (52) was treated with pyridinium chlorochromate<sup>46</sup>, an oxidant which allows the isolation of aldehydes, normally susceptible to further oxidation. The aldehyde (51), possessing the same spectroscopic properties as the synthetic racemate (Section 3.3.1) was obtained in 81% yield.

\*At this low level of  $^{18}\text{O}$ -labelling the smaller isotope-shift expected in the 2'-acetate carbonyl signal could not be resolved.



Scheme 25 Preparation of  $[1',3-^2\text{H}_4]\text{LL-D253}\alpha$

Base-catalysed deuterium exchange of the four methylene protons situated  $\alpha$  to carbonyl functions was achieved using a solution of  $\text{NaOCD}_3$  in  $\text{CD}_3\text{OD}$  and the tetradeuterated product (88) was then reduced with sodium borohydride to give LL-D253 $\alpha$ -benzyl ether (89) specifically deuterated at the C-1' and C-3 positions. This was established by both  $^1\text{H}$  and  $^2\text{H}$  n.m.r. spectroscopy (details in experimental section). Hydrogenolysis gave LL-D253 $\alpha$  (90) itself: the  $^1\text{H}$  n.m.r. spectrum confirmed that no migration of deuterium label had occurred on deprotection of the 7-hydroxyl group. Finally, heating the tetra-deuterated LL-D253 $\alpha$  (90) with a catalytic quantity of *para*-toluenesulphonic acid resulted only in the loss of about 60% of the deuterium label at C-3, presumably by acid-catalysed enolisation. The label at C-1' was unaffected. However, further work is necessary before the possibility of an *in vitro* randomisation can be discounted.

### 3.5.7 Possible Future Experiments

It would be worth investigating whether base catalysis could effect the *in vitro* transposition of deuterium label in  $[1'-^2\text{H}_2]\text{LL-D253}\alpha$ . Failing that, perhaps hydrolysis of the preformed 2'-*para*-toluenesulphonate (say) would give LL-D253 $\alpha$  exhibiting evidence of rearrangement. If an *in vitro* process cannot be demonstrated, then the labelled metabolite could be added to a mycelial suspension of *P. pigmentivora* and examined on reisolation.

Although the experiments with  $^{13}\text{C}$ -labelled acetates showed no starter effects, further evidence on this point, particularly in view of the two-chain pathway proposed above, is desirable. Labelled malonate might give a more positive result. As regards the mechanism of pyranone ring-closure, the incorporation of  $[2-^{13}\text{C}, ^2\text{H}_3]\text{acetate}$  could probably remove doubt about how many acetate hydrogens can be retained at C-3. More ambitiously, the synthesis in labelled and unlabelled form of some of the intermediates of Scheme 22 would allow a more detailed investigation of the later stages of biosynthesis.



### 3.6 Experimental

#### 3.6.1 General Procedures and Instrumentation

Melting points were determined on a Reichert hot-stage microscope and are uncorrected. Microanalyses were performed on a Perkin-Elmer 204 elemental analyser. A Varian DMS 90 spectrophotometer was used to obtain ultraviolet/visible spectra; baseline correction for solvent absorption was carried out.  $\epsilon$  is in units of  $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$  ( $\log \epsilon$  quoted below). Infra-red spectra were taken on a Perkin-Elmer 781 spectrophotometer and referenced against the polystyrene absorption at  $1601\text{ cm}^{-1}$ . Proton n.m.r. spectra were obtained from various instruments: Varian EM 360 and HA 100 continuous-wave machines and Bruker WP 80 SY, WP 200 SY, WM/WB 300 and WH 360 Fourier-transform machines. Carbon-13 n.m.r. spectra were obtained from: Varian XL 100 and CFT 20 and Bruker WP 200 SY, WH 360 and WH 400 Fourier-transform machines. Deuterium n.m.r. spectra were obtained on a Bruker WH 360, operating in this case without a frequency lock. In all cases, quoted chemical shifts are relative to tetramethylsilane,  $\delta_{\text{H}}$  and  $\delta_{\text{C}} = 0.0$  p.p.m. Mass spectra and exact mass determinations were taken on a A.E.I. MS 902 high-resolution instrument, ionising by electron impact. Peak intensities are expressed as percentages relative to the base peak at 100%.

Radiocounting was carried out using a Beckman LS 7000 liquid scintillation counter, operating on program 4 without automatic quench correction. Counting efficiency was

determined by using both standard channels ratio and H-number quench curves. The scintillant was butyl-PBD ( $10 \text{ g l}^{-1}$ ) in methanol-toluene (50:50). Samples for radiocounting were purified to constant activity by recrystallisation (except in the case of terretonin - Chapter 4) and dissolved in either methanol or toluene.

Unless otherwise specified, thin layer chromatography was performed using either analytical (5 x 20 cm) or preparative (20 x 20 cm) glass plates coated with a 0.5 mm layer of silica-gel (Merck Art. 7730 Kieselgel 60 GF<sub>254</sub> or Fluka AG 60765 Kieselgel GF 254). Where precoated metal strips are specified, Merck Art. 5548 HP-TLC-Alufolien Kieselgel 60 F 254 was used. Unless stated otherwise, ultra-violet light of wavelength 254 nm was used to visualise chromatograms.

Solutions for feeding were sterilized by autoclaving at 15 p.s.i. for 15 minutes. Dry solvents were obtained by standard procedures<sup>76</sup>.

### 3.6.2 Synthetic Experimental

#### *Crotonic anhydride*

Crotonic acid (40 g, 0.47 mol) and acetic anhydride (148 g, 1.45 mol) were heated together under reflux for 47 hours. Precautions were taken to exclude moisture. The bulk of the acetic anhydride was then removed by distillation; the concentrate was taken up in dry ether

(200 ml) and shaken with anhydrous sodium carbonate. After filtration, the ether was removed *in vacuo* and the residue was distilled under reduced pressure through a short Vigreux column. The fraction boiling between 128 and 157°C at 54 mmHg was retained. N.m.r. spectroscopy confirmed that the product was practically pure crotonic anhydride (20.1 g, 0.131 mol, 56%) (lit.<sup>77</sup>, b.p. 128-130°C at 19 mmHg).

<sup>1</sup>H N.M.R.: (60 MHz, CDCl<sub>3</sub>) δ 1.83 (3H, dd, J = 7, 1.5 Hz CH-CH<sub>3</sub>), 5.87 (1H, dq, J = 16, 1.5 Hz, CO-CH), 7.11 (1H, dq, J = 16, 7 Hz, CH-CH<sub>3</sub>)

*5,7-dihydroxy-2-methyl-4-chromanone (25)*

Anhydrous phloroglucinol (2) (7.67 g, 61 mmol) and anhydrous aluminium trichloride (25 g, 187 mmol) were stirred in dry redistilled nitrobenzene (75 ml) at room temperature until dissolved, giving a dark-green solution. Moisture was carefully excluded. Crotonic anhydride (9.44 g, 61 mmol) and a solution of anhydrous aluminium trichloride (16.4 g, 123 mmol) in nitrobenzene (60 ml) were then added simultaneously from separate dropping funnels (with stirring) over one hour. When the additions were complete the mixture was heated to 50°C and stirred at this temperature for 18 hours after which time analytical t.l.c., developing with acetone-chloroform (20:80) indicated that no phloroglucinol remained ( $R_f$  = 0.15). A major product at  $R_f$  = 0.32 was indicated.

After cooling, the mixture was decanted into a bath of iced dilute hydrochloric acid (HCl, 4N, 250 ml; ice, 250 ml). The nitrobenzene layer was separated and washed with dilute HCl (4N, 100 ml) and the combined aqueous layers were then extracted with ethyl acetate (3 x 100 ml). The extract was reduced *in vacuo* to about 200 ml and washed with dilute HCl (1N, 4 x 100 ml) to remove any remaining aluminium trichloride. The nitrobenzene was removed from the combined organic layers by prolonged steam-distillation, leaving the residual volume of the aqueous distilland at about 120 ml. This was decanted, while still hot, from a brown oil and refrigerated overnight, yielding brown crystals (1.20 g). These were purified by sublimation (170°C at 0.2 mmHg) giving an off-white crystalline sublimate (1.06 g). Analytical t.l.c. of the brown oil indicated that its major component was the same as the crystalline material. The oil was therefore purified by sublimation under the same conditions, affording further crystalline product (3.17 g): 5,7-dihydroxy-2-methyl-4-chromanone (25) (total yield 4.23 g, 22 mmol, 36%), pure by proton n.m.r. spectroscopy. Recrystallisation from aqueous ethanol gave white needles, m.p. 176-178°C (lit.<sup>29</sup>, 176-177°C).

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  3160 (vbr, m), 1633 (s), 1605 (s), 1166 (s)  $\text{cm}^{-1}$ .

<sup>1</sup>H N.M.R.: (60 MHz, CDCl<sub>3</sub>-DMSO d<sup>6</sup> (70:30))  $\delta$  1.47 (3H, d, J = 6 Hz, CH-CH<sub>3</sub>), 2.62 (2H, AB of ABX, 3-H<sub>2</sub>) 4.2-4.9 (1H, m, 2-H), 5.98 (2H, s, 2 x Ar-H), 10.36 (1H, brs, ex, 7-OH), 12.21 (1H, s, ex, 5-OH).

*7-allyloxy-5-hydroxy-2-methyl-4-chromanone (31)*

5,7-dihydroxy-2-methyl-4-chromanone (25) (1.01 g, 5.21 mmol), allyl bromide (0.50 ml, 0.71 g, 5.87 mmol) and anhydrous  $K_2CO_3$  (3.00 g) were heated in dry acetone (75 ml) under reflux with stirring. Moisture was excluded. The progress of the reaction was followed by analytical t.l.c., developing with methanol-chloroform (10:90) (starting material,  $R_f = 0.45$ ; major product,  $R_f = 0.71$ ). After 17.5 hours the flask was allowed to cool and the inorganic salts were removed by filtration and washed with further acetone. The combined filtrate and washings were reduced *in vacuo* to a brown oil which, on the addition of ice, formed pale brown crystals of *7-allyloxy-5-hydroxy-2-methyl-4-chromanone (31)* (0.77 g, 3.31 mmol, 64%). Sublimation (60°C at 0.1 mmHg) gave white needles, m.p. 55-58°C.

Found:  $M^+ = 234.0861$ .  $C_{13}H_{14}O_4$  requires  $M = 234.0892$ .

U.V.:  $\nu_{max}^{MeOH}$  (log  $\epsilon$ ) 317 (sh, 3.52), 286 (4.28), 266 (sh, 4.21), 215 (4.35) nm

I.R.:  $\nu_{max}^{KBr}$  1644 (s), 1609 (m, sh), 1575 (m), 1306 (m), 1164 (s)  $cm^{-1}$

$^1H$  N.M.R.: (60 MHz,  $CDCl_3$ )  $\delta$  1.52 (3H, d,  $J = 6$  Hz,  $CH-CH_3$ ), 2.67 (2H, AB of ABX,  $3-H_2$ ), 4.58 (2H, dm,  $J = 5$  Hz,  $O-CH_2$ ), 4.35-4.9 (1H, m,  $2-CH$ ), 5.2-5.65 (2H, m,  $CH=CH_2$ ), 5.7-6.4 (1H, m,  $CH=CH_2$ ), 6.10 (2H, AB,  $J = 2$  Hz, 2 x Ar-H), 12.04 (s, 5-OH)

M.S.: m/z (%) 234 ( $M^+$ , 100), 219 (22), 194 (14), 192 (28), 164 (16), 123 (17), 82 (17), 69 (25), 41 (53)

*7-allyloxy-5-methoxy-2-methyl-4-chromanone (27)*

7-allyloxy-5-hydroxy-2-methyl-4-chromanone (31) (582 mg, 2.49 mmol), methyl iodide (0.50 ml, 1.14 g, 8.03 mmol) and anhydrous  $K_2CO_3$  (2.06 g) were heated together in dry acetone (75 ml) under reflux with stirring. Moisture was excluded. The progress of the reaction was monitored by analytical t.l.c., developing with acetone-ethyl acetate-petroleum ether (b.p. 30-40°C) (5:45:50). The product was slightly more polar than the phenol. After 24 hours the flask was cooled; the inorganic salts were removed by filtration and washed with further acetone. The combined filtrate and washings were concentrated *in vacuo*, taken up in diethyl ether (100 ml) and washed with water (3 x 20 ml). After drying ( $MgSO_4$ ) the ether was removed to give a yellow oil (1.02 g). Preparative t.l.c., developing with ethyl acetate-diethyl ether (20:80) yielded ( $R_f = 0.8$ ) *7-allyloxy-5-methoxy-2-methyl-4-chromanone (27)* (498 mg, 2.01 mmol, 81%) as a yellow oil which crystallised on treatment with petroleum ether (b.p. 40-60°C).

(This product was also synthesised in 50% overall yield by a one-pot sequence from 5,7-dihydroxy-2-methyl-4-chromanone (25) exactly analogous to the one-pot synthesis of 5-allyloxy-7-methoxy-2-methyl-4-chromanone (26).)

An analytical sample was prepared by recrystallisation, forming white prisms from diethyl ether-hexane, m.p. 83.5-84.5°C.

Found: C, 67.68; H, 6.65.  $C_{14}H_{16}O_4$  requires C, 67.73;  
H, 6.50%

U.V.:  $\lambda_{\text{max}}^{\text{MeOH}}$  (log  $\epsilon$ ) 310 (sh, 3.62), 281 (4.25), 225 (4.25)  
213 (4.32) nm

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  1666 (s), 1610 (s), 1574 (s)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (100 MHz,  $\text{CDCl}_3$ )  $\delta$  1.43 (3H, d,  $J = 6$  Hz,  $\text{C}-\underline{\text{CH}}_3$ ),  
2.57 (2H, AB of ABX,  $3-\underline{\text{H}}_2$ ), 3.83 (3H, s,  $\text{O}-\underline{\text{CH}}_3$ ),  
4.3-4.7 (1H, m,  $2-\underline{\text{H}}$ ), 4.50 (2H, dt,  $J = 5, 1.5$  Hz,  
 $\text{O}-\underline{\text{CH}}_2-$ ), 5.29 (1H, dm,  $J = 10$  Hz,  $-\text{CH}=\underline{\text{CH}}$  *cis*),  
5.38 (1H, dm,  $J = 18$  Hz,  $-\text{CH}=\underline{\text{CH}}$  *trans*), 5.8-6.2  
(1H, m,  $-\underline{\text{CH}}=\text{CH}_2$ ) 6.04 (2H, s, 2 x Ar- $\underline{\text{H}}$ )

$^{13}\text{C}$  N.M.R.: (20 MHz,  $\text{CDCl}_3$ )  $\delta$  20.4 (q), 45.3 (t), 55.7 (q),  
68.6 (t), 73.5 (d), 92.9 (d), 93.7 (d), 105.5 (s),  
118.0 (t), 131.9 (d), 161.8 (s), 164.3 (s),  
164.7 (s), 189.3 (s)

M.S.: m/z (%) 248 ( $\text{M}^+$ , 100), 206 (29), 179 (17), 137 (20),  
69 (29), 41 (56)

*8-allyl-7-hydroxy-5-methoxy-2-methyl-4-chromanone (32)*

7-Allyloxy-5-methoxy-2-methyl-4-chromanone (27) (425 mg,  
1.71 mmol), sealed under vacuum (0.5 mmHg) in a strong  
glass tube, (8 mm i.d. x 10 cm) was heated (200°C) for  
50 minutes producing a red liquid which solidified on cooling.  
Flash chromatography to the following specification:

Column Length:	20 cm
Column Diameter:	3.5 cm
Stationary Phase:	Merck Art. 7734 Kieselgel 60, 70-230 mesh (100 g)
Mobile Phase:	acetone-chloroform (10:90)
Applied Pressure:	170 mmHg
Flow Rate:	250 ml min <sup>-1</sup>
Fraction Size:	50 ml

yielded, in fractions 10-28, *8-allyl-7-hydroxy-5-methoxy-2-methyl-4-chromanone* (32) as a clear gum which crystallized on standing (287 mg, 1.16 mmol, 68%). This compound could also be purified by preparative t.l.c., developing with acetone-chloroform, (20:80) ( $R_f = 0.31$  cf. starting allyl ether at  $R_f = 0.54$ ). Sublimation (180°C at 0.02 mmHg, 84%) gave fine white needles, m.p. 198-199.5°C (dec.).

Found: C, 67.93; H, 6.59.  $C_{14}H_{16}O_4$  requires C, 67.73; H, 6.50%

U.V.:  $\lambda_{\max}^{\text{MeOH}}$  (log  $\epsilon$ ), 315 (sh, 3.65), 286 (4.19), 232 (sh, 4.12), 214 (4.25) nm

I.R.:  $\nu_{\max}^{\text{KBr}}$  3200 (m, br), 1646 (s), 1593 (s), 1280 (m), 1122 (m) cm<sup>-1</sup>

<sup>1</sup>H N.M.R.: (80 MHz, (CD<sub>3</sub>)<sub>2</sub>CO)  $\delta$ 1.43 (3H, d, J = 6.2 Hz, C-CH<sub>3</sub>), 2.47 (2H, AB of ABX, 3-H<sub>2</sub>) 2.6-3.1 (brs, ex, 7-OH), 3.30 (2H, dt, J = 6, 1.5 Hz, O-CH<sub>2</sub>-), 3.72 (3H, s, O-CH<sub>3</sub>), 4.3-4.7 (1H, m, 2-H), 4.8-5.15 (2H, m, CH=CH<sub>2</sub>), 5.65-6.2 (1H, m, -CH=CH<sub>2</sub>), 6.19 (1H, s, Ar-H)



$^{13}\text{C}$  N.M.R.: (20 MHz,  $\text{d}^6$  DMSO)  $\delta$ 20.4 (q), 26.6 (t), 45.2 (t),  
55.3 (q), 73.4 (d), 92.6 (d), 104.5 (s), 105.5 (s),  
114.2 (t), 136.6 (d), 159.9 (s), 161.7 (s), 161.7 (s)  
188.5 (s)

M.S.: m/z (%) 248 ( $\text{M}^+$ , 100), 219 (34), 191 (28), 149 (51),  
148 (51).

Also recovered, from fractions 4-9, and initially mistaken (on the basis of t.l.c.) for the starting material with which it co-chromatographed, was an unexpected product (90 mg) tentatively identified by  $^1\text{H}$  n.m.r. spectroscopy as *5-methoxy-2,8-dimethyl-2,3,8,9-tetrahydro-4H-furo[2,3-h]-1-benzopyran-4-one* (41) (mixture of diastereoisomers).

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  1671 (s), 1623 (s), 1605 (sh, s), 1595 (sh, s),  
1131 (m)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (200 MHz,  $\text{CDCl}_3$ )  $\delta$ 1.41 (3H, d,  $J = 6.3$  Hz, 2- $\text{CH}_3$ ), 1.43 (3H, pair of d,  $\Delta\nu = 1.1$  Hz,  $J = 6.3$  Hz, 8- $\text{CH}_3$ ), 2.54 (2H, AB of ABX, 3- $\text{H}_2$ ), 2.64 (1H, pair of dd,  $\Delta\nu = 2.5$  Hz,  $J = 7.3, 15.0$  Hz, 9- $\text{H}$ ), 3.18 (1H, pair of dd,  $\Delta\nu = 1.4$  Hz,  $J = 9.0, 15.0$  Hz, 9- $\text{H}$ ), 3.81 (3H, s, O- $\text{CH}_3$ ), 4.4-4.6 (1H, m, 2- $\text{H}$ ), 4.9-5.1 (1H, m, 8- $\text{H}$ ), 5.98 (1H, s, 6- $\text{H}$ ).

*8-allyl-7-benzyl-5-methoxy-2-methyl-4-chromanone* (49)

8-Allyl-7-hydroxy-5-methoxy-2-methyl-4-chromanone (32)

(52 mg, 0.21 mmol), benzyl bromide (0.030 ml, 43 mg, 0.25 mmol) and anhydrous  $K_2CO_3$  (100 mg) were heated together in dry purified acetone (15 ml) under reflux with stirring. The progress of the reaction was followed by analytical t.l.c., developing with acetone-chloroform (20:80) (product at  $R_f$  = 0.70; starting material at  $R_f$  = 0.37). After 14 hours the flask was cooled and the insoluble inorganic salts were removed by filtration and washed with a little acetone. The filtrate and washings were reduced to a small volume, taken up in ethyl acetate (50 ml), washed with water (3 x 20 ml) and dried ( $MgSO_4$ ) to yield, on evaporation of the solvent, a pale brown oil containing benzyl bromide. Preparative t.l.c., developing with acetone-chloroform (10:90), gave on elution (ethyl acetate) of the major band at  $R_f$  = 0.6, *8-allyl-7-benzyl-5-methoxy-2-methyl-4-chromanone* (49) (71 mg, 0.21 mmol, 100%) as a very pale yellow gum. Recrystallisation from diethyl ether gave white needles, m.p. 114.5-116°C.

Found: C, 74.66; H, 6.61.  $C_{21}H_{22}O_4$  requires C, 74.54; H, 6.55%.

U.V.:  $\lambda_{max}^{MeOH}$  (log  $\epsilon$ ) 316 (sh, 3.76), 284 (4.37), 246 (4.30), 215 (4.52) nm

I.R.:  $\nu_{max}^{KBr}$  1665 (s), 1599 (s), 1575 (m), 1345 (m), 1273 (m), 1126 (s)  $cm^{-1}$

$^1H$  N.M.R.: (200 MHz,  $CDCl_3$ )  $\delta$  1.43 (3H, d,  $J$  = 6.3 Hz, C- $\underline{CH}_3$ ), 2.56 (2H, AB of ABX, 3- $\underline{H}_2$ ), 3.36 (2H, dt,  $J$  = 6.3, 1.4 Hz, 8- $\underline{CH}_2$ -), 3.83 (3H, s, O- $\underline{CH}_3$ ), 4.35-4.55

(1H, m, 2-H), 4.85-5.05 (2H, m, -CH=CH<sub>2</sub>), 5.12 (2H, s, O-CH<sub>2</sub>), 5.90 (1H, ddt, J = 17.0, 10.0, 6.3 Hz, -CH=CH<sub>2</sub>), 6.12 (1H, s, Ar-H), 7.25-7.45 (5H, m, -C<sub>6</sub>H<sub>5</sub>).

<sup>13</sup>C N.M.R.: (20 MHz, CDCl<sub>3</sub>) δ 20.5 (q), 26.8 (t), 45.5 (t), 55.7 (q), 70.0 (t), 73.4 (d), 89.6 (d), 105.8 (s), 108.4 (s), 114.0 (t), 126.8 (d), 126.8 (d), 127.9 (d), 128.4 (d), 128.4 (d), 136.2 (s), 136.2 (d), 160.5 (s), 161.3 (s), 161.9 (s), 190.2 (s).  
M.S.: m/z (%) 338 (M<sup>+</sup>, 29), 247 (12), 205 (11), 91 (100).

*7-benzyloxy-8-(2,3-dihydroxypropyl)-5-methoxy-2-methyl-4-chromanone (50)*

To an ice-cold solution of OsO<sub>4</sub> (179 mg, 0.70 mmol) in dry pyridine (2 ml), stirred under a dry N<sub>2</sub> atmosphere, was added an ice-cold solution of 8-allyl-7-benzyloxy-5-methoxy-2-methyl-4-chromanone (49) (228 mg, 0.675 mmol) in dry pyridine (4 ml). The mixture rapidly darkened and was allowed to reach room temperature. Analytical t.l.c., developing with chloroform-methanol (96:4), was used to follow the reaction. After 2 hours no alkene (R<sub>f</sub> = 0.60) remained. A solution of Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub> (282 mg, 1.48 mmol) in water (2 ml) was added and stirring was continued overnight. The flask contents were taken up in water (60 ml) and extracted with ethyl acetate (3 x 20 ml). The combined organic phases were dried (MgSO<sub>4</sub>) and the solvent was evaporated to yield *7-benzyloxy-8-(2,3-dihydroxypropyl)-5-methoxy-2-methyl-4-chromanone (50)* (mixture of diastereoisomers) as an off-white solid (235 mg, 0.632 mmol, 94%). Recrystallisation from ethyl acetate gave rosettes of needles, m.p. 150.5-152°C.

Found:  $M^+ = 372.152$ ;  $C_{21}H_{24}O_6$  requires  $M = 372.157$ .

U.V.:  $\lambda_{\max}^{\text{MeOH}}$  (log  $\epsilon$ ) 315 (sh, 3.75), 285 (4.35),  
235 (sh, 4.29), 215 (4.60) nm

I.R.:  $\nu_{\max}^{\text{KBr}}$  3360 (m, br), 1678 (s), 1602 (s), 1380 (s),  
1351 (m), 1126 (s)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (360 MHz,  $\text{CDCl}_3$ )  $\delta$  1.45 (3H, pair of d,  $\Delta\nu = 0.6$  Hz,  
 $J = 6.3$  Hz,  $\text{C}-\text{CH}_3$ ), 2.0-2.5 (brs, ex,  $\text{OH}$ ),  
2.57 (2H, AB of ABX,  $3-\text{H}_2$ ), 2.77-2.93 (2H, pair,  
 $\Delta\nu = 6.5$  Hz, of AB of ABX, 16 lines,  $J_{\text{AB}} = 13.5$  Hz,  
 $8-\text{CH}_2$ ), 3.40-3.56 (2H, pair,  $\Delta\nu = 2.6$  Hz, of AB of  
ABX, 14 lines,  $J_{\text{AB}} = 11.5$  Hz,  $-\text{CH}_2\text{OH}$ ), 3.81-3.92  
(1H, m,  $-\text{CHOH}-$ ), 3.83 (3H, s,  $\text{O}-\text{CH}_3$ ), 4.42-4.53  
(1H, m,  $2-\text{H}$ ), 5.12 (2H, s,  $\text{Ph}-\text{CH}_2-$ ), 6.14 (1H, s,  
 $6-\text{H}$ ), 7.29-7.42 (5H, m,  $-\text{C}_6\text{H}_5$ )

$^{13}\text{C}$  N.M.R.: (20 MHz,  $\text{CDCl}_3$ )  $\delta$  20.5 (q), 26.6 (t), 45.5 (t),  
55.8 (q), 65.8 (t), 70.4 (t), 71.7 (d), 73.8 (d),  
89.9 (d), 105.9 (s), 106.1 (s), 127.1 (d), 127.1 (d),  
128.3 (d), 128.7 (d), 128.7 (d), 136.6 (s), 160.9 (s),  
161.5 (s), 162.3 (s), 189.8 (s)

M.S.: m/z (%) 372 ( $M^+$ , 16), 311 (31), 221 (11), 91 (100),  
43 (56).

*7-benzyloxy-5-methoxy-2-methyl-8-(2-oxoethyl)-4-chromanone (51)*

Method A: To a solution of 7-benzyloxy-8-(2,3-dihydroxypropyl)-5-methoxy-2-methyl-4-chromanone (50) (160 mg, 0.430 mmol) in methanol (7 ml) was added a solution of  $\text{NaIO}_4$  (109 mg, 0.510 mmol) in water (7 mls). The mixture became slightly clouded and was stirred at room temperature for 15 hours. Analytical t.l.c., developing with methanol-chloroform (4:96); product  $R_f = 0.45$ , diol  $R_f = 0.30$ , indicated that the reaction was complete. Water (50 ml) was added and the total was extracted with ethyl acetate (5 x 30 ml). After drying ( $\text{MgSO}_4$ ) the solvent was removed *in vacuo* to yield *7-benzyloxy-5-methoxy-2-methyl-8-(2-oxoethyl)-4-chromanone (51)* (161 mg, 0.474, 110%) as a white, crystalline mass. A single recrystallisation from ethyl acetate-petroleum ether (b.p. 40-60°C) gave, in small yield, irregular white crystals (40 mg), m.p. 123-127°C. Further attempts at recrystallisation resulted in decomposition and the compound also proved fairly unstable to preparative t.l.c.

Method B: 7-benzyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (LL-D253 $\alpha$  - benzyl ether) (52) (51 mg, 0.149 mmol) dissolved in dry methylene chloride (1 ml) was added to a suspension of pyridinium chlorochromate (48 mg, 0.223 mmol) in dry methylene chloride (0.5 ml) and the mixture was stirred at room temperature. Moisture was excluded. The mixture slowly darkened and the reaction was monitored by analytical t.l.c., developing with methanol-

chloroform (4:96), product  $R_f = 0.46$ , starting alcohol  $R_f = 0.22$ . After 2 hours, a spot at  $R_f = 0.22$  was still visible and further pyridinium chlorochromate (10 mg, 0.046 mmol) was added. At 4 hours, careful examination of the t.l.c. plate under both long (350 nm) and short (254 nm) wavelength u.v. light indicated that the remaining material at  $R_f = 0.22$  was not the starting alcohol. The reaction mixture was diluted with dry diethyl ether (10 ml) resulting in the formation of black insoluble material from which the supernatant liquor was decanted. The black solid was washed with further volumes of dry ether (causing it to become granular in appearance) and the combined supernatant and washings were filtered through Celite to give a clear solution which, on removal *in vacuo* of the solvents, yielded 7-benzoyloxy-5-methoxy-2-methyl-8-(2-oxoethyl)-4-chromanone (51) (41 mg, 0.121 mmol, 81%) as a white crystalline solid.

Found:  $M^+ = 340.131$ ,  $C_{20}H_{20}O_5$  requires  $M = 340.131$

U.V.:  $\lambda_{\text{max}}^{\text{CHCl}_3}$  (log  $\epsilon$ ) 310 (3.63), 283 (4.24), 252 (4.01) nm

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  1724 (m), 1677 (s), 1602 (s), 1580 (sh, m), 1154 (m), 1123 (s)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (60 MHz,  $\text{CDCl}_3$ )  $\delta$  1.45 (3H, d,  $J = 6$  Hz, C- $\text{CH}_3$ ), 2.60 (2H, AB of ABX, 3- $\text{H}_2$ ), 3.67 (2H, d,  $J = 1.5$  Hz, Ar- $\text{CH}_2$ -), 3.87 (3H, s, -O- $\text{CH}_3$ ), 4.2-4.8 (1H, m, 2- $\text{H}$ ), 5.14 (2H, s, -O- $\text{CH}_2$ -), 6.18 (1H, s, Ar- $\text{H}$ ), 7.36 (5H, s, - $\text{C}_6\text{H}_5$ ), 9.60 (1H, t,  $J = 1.5$  Hz, -CHO)

$^{13}\text{C}$  N.M.R.: (50 MHz,  $\text{CDCl}_3$ )  $\delta$  20.4 (q), 37.8 (t), 45.5 (t),  
55.9 (q), 70.5 (t), 74.0 (d), 90.0 (d),  
101.7 (s), 106.0 (s), 127.1 (d), 127.1 (d),  
128.2 (d), 128.6 (d), 128.6 (d), 135.7 (s),  
161.7 (s), 161.7 (s), 162.4 (s), 189.5 (s),  
199.4 (d)

M.S.: m/z (%) 340 ( $\text{M}^+$ , 2), 312 (11), 221 (5), 179 (19),  
149 (16), 91 (76), 43 (100)

*7-benzyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (LL-D253a - benzyl ether) (52)*

Method A: A solution of sodium borohydride (10 mg, 0.26 mmol) in dry ethanol (5 ml) was prepared (by stirring at room temperature for one hour) and added dropwise, with stirring, at room temperature, over 20 minutes to a solution of 7-benzyloxy-5-methoxy-2-methyl-8-(2-oxoethyl)-4-chromanone (51) (40 mg, 0.12 mmol) in dry tetrahydrofuran (4 ml). Precautions were taken to exclude moisture. The progress of the reaction was followed by analytical t.l.c., using precoated metal strips and developing with methanol-chloroform (4:96) (product  $R_f$  = 0.43, substrate  $R_f$  = 0.58), which indicated completion after the 20 minutes. The reaction was quenched by pouring the mixture into dilute, aqueous HCl (20 ml, 0.2 N) and the resulting solution was extracted with ethyl acetate (3 x 10 ml). After drying ( $\text{MgSO}_4$ ) and evaporation of the solvent, the pale yellow gum (46 mg) thus obtained was purified by preparative

t.l.c.: 2 developments with methanol-chloroform (2:98). The band at  $R_f = 0.62$  was eluted (ethyl acetate) to give a clear gum (29 mg, 0.084 mmol, 72%).

*7-benzyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone* (52) prepared in this way recrystallised from ethyl acetate to give short white rods, m.p. 133-135°C, m.m.p. 133.5-136°C, and showed identical characteristics: (m.s., i.r.,  $^1\text{H}$  n.m.r.) to material prepared by the benzylation of natural LL-D253 $\alpha$  (see below).

Method B: 7-hydroxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (LL-D253 $\alpha$ ) (16) (265 mg, 1.05 mmol), benzyl bromide (0.135 ml, 194 mg, 1.13 mmol) and anhydrous potassium carbonate (402 mg) in dry purified acetone (25 ml) were heated together under reflux with stirring for 18 hours. Precautions were taken to exclude moisture. Analytical t.l.c., developing with methanol-chloroform (4:96), product  $R_f = 0.30$ , substrate  $R_f = 0.15$ , showed that no substrate remained. After cooling, the supernatant solution was decanted and the inorganic residue was washed with further acetone. The supernatant and washings were concentrated *in vacuo* to a small volume, taken up in ethyl acetate (50 ml) and washed with water (3 x 20 ml) which was then back-extracted with further ethyl acetate (2 x 20 ml). The combined, dried ( $\text{MgSO}_4$ ), organic layers were reduced *in vacuo* to a pale yellow oil (386 mg) containing benzyl bromide and which was purified by preparative t.l.c., developing with methanol-chloroform (4:96). The major band at  $R_f = 0.56$  was eluted (methanol-ethyl acetate (2:98)) to give, on evaporation



of the solvent, 7-benzyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (52) (312 mg, 0.91 mmol, 87%) as a clear semi-crystalline gum.  $^1\text{H}$  n.m.r. spectroscopy indicated the presence of a trace of ethyl acetate. Recrystallisation from ethyl acetate yielded short white rods, m.p. 135-136.5°C.

Found: C, 70.11; H, 6.47.  $\text{C}_{20}\text{H}_{22}\text{O}_5$  requires C, 70.17; H, 6.48%

U.V.:  $\lambda_{\text{max}}^{\text{MeOH}}$  (log  $\epsilon$ ) 316 (sh, 3.74), 284 (4.33), 234 (sh, 4.26), 213 (4.53) nm

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  3475 (br, m), 1654 (m), 1600 (s), 1575 (m), 1126 (m)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (60 MHz,  $\text{CDCl}_3$ )  $\delta$  1.47 (3H, d,  $J = 6$  Hz, C- $\text{CH}_3$ ), 1.85-2.1 (brs, OH), 2.68 (2H, AB of ABX, 3- $\text{H}_2$ ), 2.95 (2H, t,  $J = 6$  Hz, Ar- $\text{CH}_2$ -), 4.2-4.8 (1H, m, 2- $\text{H}$ ), 5.16 (2H, s, Ph- $\text{CH}_2$ -), 6.16 (1H, s, Ar- $\text{H}$ ), 7.40 (5H, s,  $-\text{C}_6\text{H}_5$ )

$^{13}\text{C}$  N.M.R.: (20 MHz,  $\text{CDCl}_3$ )  $\delta$  20.6 (q), 26.2 (t), 44.5 (t), 55.8 (q), 62.2 (t), 70.2 (t), 73.6 (d), 89.8 (d), 105.9 (s), 106.9 (s), 127.0 (d), 127.0 (d), 128.1 (d), 128.6 (d), 128.6 (d), 135.9 (s), 160.7 (s), 161.7 (s), 162.4 (s), 190.1 (s)

M.S.: m/z (%), 342 ( $\text{M}^+$ , 30), 311 (37), 221 (22), 179 (10), 91 (100), 43 (55).

*7-hydroxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (LL-D253α) (16)*

A degassed solution of 7-benzyloxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (LL-D253α - benzyl ether) (52) (278 mg, 0.81 mmol) in ethyl acetate (25 ml, redistilled) also containing in suspension, carbon-supported palladium catalyst (Pd/C, 10:90 w/w, 50 mg) was stirred under hydrogen gas at room temperature and pressure. Precautions were taken to exclude moisture. Samples were withdrawn at intervals by syringe through a rubber septum and analysed by t.l.c.: precoated metal strips, developing with methanol-chloroform (4:96), substrate  $R_f = 0.5$ , product  $R_f = 0.25$ . After 7.5 hours, t.l.c. indicated completion: the catalyst was filtered off, and the solvent was removed *in vacuo*. The resulting gum (221 mg) was purified by preparative t.l.c., developing with methanol-chloroform (10:90), to yield starting material (24 mg, 0.070 mmol) and *7-hydroxy-8-(2-hydroxyethyl)-5-methoxy-2-methyl-4-chromanone (16)* (129 mg, 0.51 mmol, 63% (69%)) as a white crystalline solid.

Material prepared in this way recrystallised from ethyl acetate - methanol to give white needles, m.p. 189-191°C, and showed identical characteristics (m.s., i.r.,  $^1\text{H}$  n.m.r., t.l.c.) to natural LL-D253α (see Section 3.6.3).

*Crotonyl Chloride*

Crotonic acid (30 g, 0.35 mol) and thionyl chloride (57 g, 0.48 mol) in dry petroleum ether (b.p. 40-60°C) (300 ml) were heated together under gentle reflux for four hours. Precautions were taken to exclude moisture. A large volume of HCl gas was evolved. The bulk of the solvent was removed *in vacuo* and the concentrated product was distilled through a short Vigreux column to give crotonyl chloride (27.7 g, 0.265 mol, 76%) as a clear liquid, b.p. 124-126°C at 760 mmHg (lit.<sup>78</sup>, 124-126°C).

<sup>1</sup>H N.M.R.: (60 MHz, CDCl<sub>3</sub>) δ2.03 (3H, dd, J = 7, 1.5 Hz, CH-CH<sub>3</sub>), 6.10 (1H, dq, J = 15, 1.5 Hz, CO-CH), 7.25 (1H, dq, J = 15, 7 Hz, CH-CH<sub>3</sub>)

*3,5-dimethoxyphenyl crotonate (54)*

3,5-dimethoxyphenol (10.00 g, 64.9 mmol) and metallic magnesium (turnings, 0.31 g) were heated together under reflux in dry benzene (30 ml) with stirring. Precautions were taken to exclude moisture. Crotonyl chloride (7.00 g, 67.0 mmol) was added dropwise over one hour resulting in the copious evolution of HCl gas. A small quantity of red solid formed as heating was continued. After 15 hours the mixture was allowed to cool. The solution was decanted from the magnesium turnings, diluted with further benzene (100 ml), washed with 1% w/v aqueous sodium hydroxide (3 x 50 ml) and water (3 x 50 ml),

dried ( $\text{CaCl}_2$ ) and reduced *in vacuo* to give the *ester* (54) as a pale yellow oil (14 g). This was distilled under reduced pressure of dry nitrogen through a short Vigreux column: the fraction boiling in the range 138-141°C (at 0.7 mmHg) was collected as a pale yellow, pleasant smelling oil (11.19 g, 50.4 mmol, 78%) which solidified on prolonged standing in a cold room. Exposure to air or light resulted in gradual darkening of the product.

Found:  $M^+ = 222.088$ ,  $\text{C}_{12}\text{H}_{14}\text{O}_4$  requires  $M = 222.089$ .

U.V.:  $\lambda_{\text{max}}^{\text{MeOH}}$  (log  $\epsilon$ ), 269 (3.27), 213 (4.32) nm

I.R.:  $\nu_{\text{max}}^{\text{neat}}$  1740 (s), 1615 (s), 1478 (m), 1156 (s), 1598 (sh, s)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (60 MHz,  $\text{CDCl}_3$ )  $\delta$  1.98 (3H, dd,  $J = 7, 1.5$  Hz,  $\text{CH}-\text{CH}_3$ ), 3.81 (6H, s, 2 x  $\text{O}-\text{CH}_3$ ), 6.08 (1H, dq,  $J = 15, 1.5$  Hz,  $-\text{CO}-\text{CH}$ ), 6.38 (3H, s, 3 x  $\text{Ar}-\text{H}$ ), 7.27 (1H, dq,  $J = 15, 7$  Hz,  $\text{CH}-\text{CH}_3$ )

$^{13}\text{C}$  N.M.R.: (50 MHz,  $\text{CDCl}_3$ )  $\delta$  17.8 (q), 55.2 (q), 55.2 (q), 98.1 (d), 100.2 (d), 100.2 (d), 122.0 (d), 146.5 (d), 152.3 (s), 161.0 (s), 161.0 (s), 164.3 (s)

M.S.:  $m/z$  (%), 222 ( $M^+$ , 47), 194 (5), 154 (90), 125 (19), 69 (100), 41 (38)

*5,7-dimethoxy-2-methyl-4-chromanone (55)*

3,5-dimethoxyphenyl crotonate (54) (2.02 g, 9.10 mmol) in benzene (200 ml) and 10% w/v aqueous sodium hydroxide (50 ml) were irradiated together overnight in a pyrex photochemical reaction vessel, fitted with a quartz, water-cooled, immersion well and a 400 W, low pressure, mercury lamp. The two solutions were mixed by the passage of benzene-saturated nitrogen through a glass sinter in the base of the vessel. The reaction did not go to completion: analytical t.l.c., developing with acetone-chloroform (5:95), showed the presence of both starting material ( $R_f = 0.64$ ) and product ( $R_f = 0.36$ ).

After three such identical procedures, the benzene component of the three combined reaction mixtures was separated and the basic layer was extracted with further benzene (2 x 200 ml). The combined benzene layers were washed with water (2 x 200 ml), dried ( $\text{CaCl}_2$ ), and reduced *in vacuo* to a yellow gum (3.3 g). This was subjected to flash-chromatography to the following specification:

Stationary Phase:	Fluka AG 60765 Kieselgel GF 254 (160 g)
Mobile Phase:	Diethyl ether
Column Length:	15 cm
Column Diameter:	5 cm i.d.
Fraction size:	100 ml
Applied Pressure:	200 mmHg

On the basis of t.l.c., fractions 8-16 were combined and concentrated to give 5,7-dimethoxy-2-methyl-4-chromanone (55) as a clear gum (1.28 g, 5.77 mmol, 21%) which crystallised to a white mass on standing. Recrystallisation from ether gave rosettes of rods, m.p. 80-80.5°C (lit.<sup>50</sup>, 77-79°C).

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  1675 (s), 1609 (s), 1572 (m), 1465 (m),  
808 (m)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (60 MHz,  $\text{CDCl}_3$ )  $\delta$  1.45 (3H, d,  $J = 6$  Hz, C- $\text{CH}_3$ ),  
2.58 (2H, AB of ABX, 3- $\text{H}_2$ ), 3.78 (3H, s, O- $\text{CH}_3$ ),  
3.84 (3H, s, O- $\text{CH}_3$ ), 4.2-4.7 (1H, m, 2- $\text{H}$ ),  
6.00 (2H, s, 2 x Ar- $\text{H}$ )

M.S.: m/z (%), 222 ( $\text{M}^+$ , 93), 193 (16), 180 (100), 152 (28),  
137 (28)

*5-hydroxy-7-methoxy-2-methyl-4-chromanone (53)*

To a solution of 5,7-dimethoxy-2-methyl-4-chromanone (55) (1.28 g, 5.74 mmol) in dry methylene chloride (50 ml), at -78°C, was added boron trichloride (2 ml, 2.9 g, 25 mmol), also previously chilled to -78°C. A dark orange colouration developed immediately. The flask was sealed and left at room temperature for 30 minutes with occasional agitation. After rechilling, the flask was opened and attached to a rotary evaporator (no heat applied) for 30 minutes to remove the excess  $\text{BCl}_3$ .

The dark solution was then diluted (to 100 ml) with further methylene chloride, washed with water (4 x 30 ml) and dried ( $\text{MgSO}_4$ ), and the solvent was removed by gentle evaporation under reduced pressure (no heat applied). This yielded *5-hydroxy-7-methoxy-2-methyl-4-chromanone* (53) (1.15 g, 5.53 mmol, 96%) as a translucent, slightly green gum, pure by  $^1\text{H}$  n.m.r. spectroscopy, which was stored in the dark under nitrogen. On prolonged exposure to air, or on attempts to crystallise the gum, the green colour intensified markedly. Recrystallisation from ethyl acetate-hexane gave dark-green crystalline lumps which, however, melted sharply, m.p. 86.5-87.5°C (sub.) to a colourless melt. Sublimation (80°C at 0.2 mmHg) gave the product as white needles, m.p. 87-88°C (sub.).

Found: C, 63.26; H, 5.78.  $\text{C}_{11}\text{H}_{12}\text{O}_4$  requires C, 63.46; H, 5.81.

U.V.:  $\lambda_{\text{max}}^{\text{MeOH}}$  (log  $\epsilon$ ), 322 (3.46), 296 (4.24), 226 (sh, 4.15), 213 (4.29) nm.

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  1640 (br, s), 1580 (s), 1304 (m), 1290 (m), 1211 (s), 1154 (s)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (60 MHz,  $\text{CDCl}_3$ )  $\delta$  1.50 (3H, d,  $J = 6$  Hz, C- $\text{CH}_3$ ), 2.62 (2H, AB of ABX, 3- $\text{H}_2$ ), 3.79 (3H, s, O- $\text{CH}_3$ ), 4.3-4.8 (1H, m, 2- $\text{H}$ ), 5.97 (2H, AB,  $J = 2.5$  Hz, 2 x Ar- $\text{H}$ ), 12.13 (1H, s, Ar-OH)

$^{13}\text{C}$  N.M.R.: (50 MHz,  $\text{CDCl}_3$ )  $\delta$  20.6 (q), 43.3 (t), 55.4 (q), 73.8 (d), 93.8 (d), 94.7 (d), 103.0 (s), 162.9 (s), 164.1 (s), 167.8 (s), 196.0 (s)

M.S.: m/z (%) 208 ( $\text{M}^+$ , 100), 193 (31), 167 (46), 166 (100), 138 (44), 95 (29)

*5-allyloxy-7-methoxy-2-methyl-4-chromanone* (26)

Method A: 5,7-dihydroxy-2-methyl-4-chromanone (25) (1.002 g, 5.16 mmol), methyl iodide (0.32 ml, 0.730 g, 5.14 mmol) and anhydrous potassium carbonate (3.01 g) were heated together under reflux in dry purified acetone (100 ml) with stirring. The progress of the reaction was monitored by analytical t.l.c., developing with acetone-chloroform (20:80). After 17 hours no starting material ( $R_f = 0.50$ ) was present and one major product was indicated ( $R_f = 0.66$ ). Allyl bromide (2.2 ml, 3.12 g, 25.8 mmol) was added and heating was continued. At 23 hours further  $K_2CO_3$  (3.00 g) was added as the second reaction (giving one major product at  $R_f = 0.50$ ) was proceeding only slowly. The reaction mixture was allowed to cool at 72 hours.

The inorganic salts were removed by filtration and washed with acetone. The combined filtrate and washings were reduced *in vacuo* to a semicrystalline gum, taken up in ethyl acetate (100 ml), washed with water (1 x 100 ml, 3 x 50 ml) and dried ( $MgSO_4$ ). Removal of the solvent (*in vacuo*) yielded a brown gum, the constituents of which were separated by preparative t.l.c. (developing with diethyl ether). The band at  $R_f = 0.8$  was eluted (ethyl acetate) and afforded *5-allyloxy-7-methoxy-2-methyl-4-chromanone* (26) (790 mg, 3.19 mmol, 62%) as a pale brown crystalline solid. Recrystallisation from diethyl ether-hexane yielded white needles, m.p. 60.5-61°C.



Method B: This material was also prepared, also in 62% yield, (using the same reagents, conditions and isolation procedure as for the second reaction of the above sequence) from 5-hydroxy-7-methoxy-2-methyl-4-chromanone (53) previously isolated.

Found: C, 67.92; H, 6.52.  $C_{14}H_{16}O_4$  requires C, 67.73; H, 6.50

U.V.:  $\lambda_{\max}^{\text{MeOH}}$  (log  $\epsilon$ ), 310 (sh, 3.62), 280 (4.22), 225 (4.26), 212 (4.28) nm

I.R.:  $\nu_{\max}^{\text{KBr}}$  1680 (s), 1611 (s), 1573 (m), 808 (m)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (200 MHz,  $\text{CDCl}_3$ )  $\delta$ 1.42 (3H, d,  $J = 6.2$  Hz, C- $\text{CH}_3$ ), 2.46-2.65 (2H, AB of ABX, 3- $\text{H}_2$ ), 3.76 (3H, s, O- $\text{CH}_3$ ), 4.38-4.58 (1H, m, 2- $\text{H}$ ), 4.53 (2H, dt,  $J = 4.7, 1.7$  Hz, Ar- $\text{CH}_2$ ), 5.28 (1H, dm,  $J = 10.6$  Hz, -CH=CH *cis*), 5.60 (1H, dm,  $J = 17.1$  Hz, -CH=CH *trans*), 5.92-6.14 (1H, m, -CH=CH<sub>2</sub>), 6.00 (2H, AB, 2 x Ar- $\text{H}$ )

$^{13}\text{C}$  N.M.R.: (20 MHz,  $\text{CDCl}_3$ )  $\delta$ 20.4 (q), 45.4 (t), 55.1 (q), 68.9 (t), 73.5 (d), 93.1 (d), 93.5 (d), 105.7 (s), 117.0 (t), 131.9 (d), 160.6 (s), 164.6 (s), 165.2 (s), 188.9 (s)

M.S.: m/z (%), 248 ( $\text{M}^+$ , 100), 233 (34), 219 (45), 150 (69), 41 (94).

*6-allyl-5-hydroxy-7-methoxy-2-methyl-4-chromanone* (56)

*8-allyl-5-hydroxy-7-methoxy-2-methyl-4-chromanone* (57)

*5-allyloxy-7-methoxy-2-methyl-4-chromanone* (26),

(902 mg, 3.64 mmol), sealed under vacuum (0.1 mmHg) in a strong pyrex tube (8 mm i.d. x 10 cm), was heated (200°C) for 25 minutes. A brown gum formed and, on opening the tube, an unpleasant, "fishy" odour was evident. Close examination revealed that the tube had cracked under heating and admitted air. A  $^1\text{H}$  n.m.r. spectrum indicated that no allyl ether remained and that two major products had been formed.

After considerable effort the products were separated by preparative t.l.c., developing with ethyl acetate-petroleum ether (b.p. 30-40°C) (6:94), three passes per plate). The slightly more polar band ( $R_f = 0.44$ ) was eluted (chloroform) to give *6-allyl-5-hydroxy-7-methoxy-2-methyl-4-chromanone* (56) (417 mg, 1.68 mmol, 46%) as a pale yellow gum. Recrystallisation from diethyl ether-petroleum ether (b.p. 30-40°C) yielded yellow crystals which, on sublimation (75°C at 0.05 mmHg), became white needles, m.p. 61-64°C.

Found: C, 67.51; H, 6.75.  $\text{C}_{14}\text{H}_{16}\text{O}_4$  requires C, 67.73; H, 6.50

U.V.:  $\lambda_{\text{max}}^{\text{MeOH}}$  (log  $\epsilon$ ), 331 (3.46), 288 (4.28), 228 (sh, 4.27), 214 (4.38), 205 (sh, 4.34) nm

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  1647 (s), 1619 (s), 1576 (m), 1299 (s), 1136 (s)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (80 MHz,  $\text{CDCl}_3$ )  $\delta$ 1.47 (3H, d,  $J = 6.3$  Hz, CH-CH $\underline{\text{H}}_3$ ), 2.43-2.90 (2H, AB of ABX,  $J_{\text{AB}} = 17.1$  Hz, 3-H $\underline{\text{H}}_2$ ), 3.28 (2H, dt,  $J = 6.0, 1.5$  Hz, Ar-CH $\underline{\text{H}}_2$ ), 3.81 (3H, s, O-CH $\underline{\text{H}}_3$ ), 4.28-4.75 (1H, m, 2-H), 4.80-5.15 (2H, m, CH=CH $\underline{\text{H}}_2$ ), 5.65-6.18 (1H, m, CH=CH $\underline{\text{H}}_2$ ), 5.99 (1H, s, Ar-H), 12.07 (1H, s, Ar-OH)

$^{13}\text{C}$  N.M.R.: (50 MHz,  $\text{CDCl}_3$ )  $\delta$ 20.7 (q), 25.9 (t), 43.4 (t), 55.7 (q), 73.9 (d), 90.6 (d), 102.8 (s), 107.9 (s), 114.0 (t), 136.2 (d), 160.4 (s), 161.8 (s), 165.4 (s), 196.1 (s)

M.S.: m/z (%), 248 (100), 233 (41), 221 (18), 205 (15), 206 (18), 179 (18), 178 (20), 69 (16)

Elution (chloroform) of the less polar band ( $R_f = 0.53$ ) gave *8-allyl-5-hydroxy-7-methoxy-2-methyl-4-chromanone* (57) (224 mg, 0.90 mmol, 25%) as a white crystalline solid. Recrystallisation from ether-hexane gave white needles, m.p. 76.5-77.5°C (sub.). Sublimation (75°C at 2.5 mmHg) raised the m.p. to 77-78°C (sub.).

Found: C, 67.52; H, 6.74.  $\text{C}_{14}\text{H}_{16}\text{O}_4$  requires C, 67.73; H, 6.50

U.V.:  $\lambda_{\text{max}}^{\text{MeOH}}$  (log  $\epsilon$ ) 334 (3.51), 289 (4.24), 236 (sh, 4.09), 214 (4.35) nm

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  1635 (s), 1585 (m), 1358 (m), 1304 (m), 1206 (m), 1156 (m)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (80 MHz,  $\text{CDCl}_3$ )  $\delta$  1.47 (3H, d,  $J = 6.3$  Hz,  $\text{CH}-\text{CH}_3$ ), 2.44-2.88 (2H, AB of ABX,  $J_{\text{AB}} = 17$  Hz, 3- $\text{H}_2$ ), 3.25 (2H, dt,  $J = 6.1, 1.5$  Hz,  $\text{Ar}-\text{CH}_2$ ), 3.83 (3H, s,  $\text{O}-\text{CH}_3$ ), 4.19-4.74 (1H, m, 2- $\text{H}$ ), 4.78-5.09 (2H, m,  $\text{CH}=\text{CH}_2$ ), 5.59-6.13 (1H, m,  $\text{CH}=\text{CH}_2$ ), 6.04 (1H, s,  $\text{Ar}-\text{H}$ ), 12.14 (1H, s,  $\text{Ar}-\text{OH}$ ).

$^{13}\text{C}$  N.M.R.: (50 MHz,  $\text{CDCl}_3$ )  $\delta$  20.6 (q), 26.4 (t), 43.3 (t), 55.7 (q), 73.6 (d), 92.1 (d), 102.8 (s), 106.9 (s), 113.9 (t), 136.4 (d), 159.2 (s), 162.8 (s), 165.5 (s), 196.6 (s)

M.S.: 248 ( $\text{M}^+$ , 100), 233 (14), 221 (19), 207 (11), 179 (19), 178 (35)

This pyrolysis was also carried out on a smaller scale (50 mg) heating ( $200^\circ\text{C}$ ) for 15 minutes. The vacuum (0.2 mmHg) was maintained throughout heating on this occasion. A  $^1\text{H}$  n.m.r. spectrum indicated that one product only had been formed, in quantitative yield, and analytical t.l.c. showed that this was the more polar of the two rearrangement products recorded above. The assignment of the respective structures for these two compounds is based on a series of specific  $^1\text{H}$  decoupling experiments on the  $^1\text{H}$ -coupled  $^{13}\text{C}$  n.m.r. spectra of the hydroxyethyl derivatives (see Section 3.3.3).

*5-hydroxy-6-(2,3-dihydroxypropyl)-7-methoxy-2-methyl-4-chromanone (58)*

To an ice-cold solution of osmium tetroxide (49 mg, 0.193 mmol) in dry pyridine (1 ml), stirred under a dry nitrogen atmosphere, was added an ice-cold solution of 6-allyl-5-hydroxy-7-methoxy-2-methyl-4-chromanone (56) (47 mg, 0.190 mmol) in pyridine (2 ml). The mixture rapidly darkened and was stirred at room temperature for 90 minutes, after which time analytical t.l.c., developing with acetone-chloroform (5:95) showed that no alkene ( $R_f = 0.35$ ) remained. One major product ( $R_f = 0.19$ ), presumably the osmate ester, was present. A solution of sodium metabisulphite (80 mg, 0.42 mmol) in water (1 ml) was added; stirring was continued for another two hours. The mixture lost its dark, opaque appearance to form a translucent orange solution above a dark brown solid. Water (20 ml) was added and the resulting solution was extracted with ethyl acetate (3 x 10 ml). The combined organic phases were dried ( $MgSO_4$ ) and the solvents were removed *in vacuo* (the pyridine was co-distilled with carbon tetrachloride) to yield *5-hydroxy-6-(2,3-dihydroxypropyl)-7-methoxy-2-methyl-4-chromanone (58)* (mixture of diastereoisomers) as a white crystalline solid. Recrystallisation from  $CDCl_3$  gave white needles, m.p. 149-152°C (with very extensive sublimation) and this melting range could not be improved by further recrystallisation.

Found:  $M^+ = 282.1085$ .  $C_{14}H_{16}O_6$  requires  $M = 282.1103$

U.V.:  $\lambda_{\text{max}}^{\text{MeOH}}$  (log  $\epsilon$ ), 332 (3.39), 287 (4.19), 215 (4.31) nm

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  3350 (br, m), 1641 (s), 1296 (s), 1213 (m)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (360  $\text{MHz}$ ,  $\text{CDCl}_3$ )  $\delta$  1.49 (3H, d,  $J = 6.3$  Hz, CH-CH $\underline{\text{H}}_3$ ), 1.59 (br s, ex, OH), 2.38 (br t,  $J = 6$  Hz, ex, OH), 2.49 (br d,  $J = 6$  Hz, ex, OH), 2.58-2.73 (2H, AB of ABX,  $J_{\text{AB}} = 17.1$  Hz, 3-CH $\underline{\text{H}}_2$ ), 2.76-2.87 (2H, m, 11 lines, Ar-CH $\underline{\text{H}}_2$ ), 3.43-3.60 (2H, br m, sharpened by  $\text{D}_2\text{O}$  to 16 lines, -CH $\underline{\text{H}}_2$ OH), 3.84 (3H, s, O-CH $\underline{\text{H}}_3$ ), 3.82-3.93 (1H, m, -CH $\underline{\text{H}}$ OH-), 4.49-4.61 (1H, m, 12 lines, 2-CH), 6.03 (1H, s, Ar-H), 12.38 (1H, 'd', ex, Ar-OH)

$^{13}\text{C}$  N.M.R.: (50  $\text{MHz}$ ,  $\text{CDCl}_3$ )  $\delta$  20.6 (q), 25.7 (t), 43.2 (t), 55.8 (q), 65.8 (t), 71.7 (d), 74.0 (d), 91.0 (d), 102.7 (s), 105.7 (s), 160.6 (s), 162.1 (s), 165.6 (s), 196.3 (s)

M.S.: m/z (%), 282 ( $M^+$ , 12), 251 (14), 221 (100), 179 (48), 69 (16)

*5-hydroxy-7-methoxy-2-methyl-6-(2-oxoethyl)-4-chromanone (60)*

5-hydroxy-6-(2,3-dihydroxypropyl)-7-methoxy-2-methyl-4-chromanone (58) (48 mg, 0.17 mmol), dissolved in methanol (2 ml), was added to a solution of sodium periodate (43 mg, 0.20 mmol) in water (1 ml) to form a cloudy suspension. The mixture was stirred at room temperature for 17 hours after which time analytical t.l.c., developing with acetone-chloroform (5:95), showed that no diol ( $R_f = 0.06$ ) remained and one product ( $R_f = 0.62$ ) was present. After dilution with water (40 ml), the reaction mixture was extracted with ethyl acetate (4 x 10 ml). The organic extract was dried ( $MgSO_4$ ) and reduced *in vacuo* to give *5-hydroxy-7-methoxy-2-methyl-6-(2-oxoethyl)-4-chromanone (60)* as an off-white crystalline mass (42 mg, 0.17 mmol, 100%). Recrystallisation from ethyl acetate-hexane yielded fine white needles, m.p. 97-99.5°C.

Found:  $M^+ = 250.0837$ .  $C_{13}H_{14}O_5$  requires  $M = 250.0841$   
 U.V.:  $\lambda_{max}^{CHCl_3}$  (log  $\epsilon$ ) 330 (3.44), 289 (4.25), 241 (3.80) n.  
 I.R.:  $\nu_{max}^{KBr}$  1726 (s), 1640 (s), 1577 (m), 1298 (s),  
 1209 (s)  $cm^{-1}$

$^1H$  N.M.R.: (200 MHz,  $CDCl_3$ )  $\delta$  1.48 (3H, d,  $J = 6.3$  Hz, C- $\underline{CH}_3$ ), 2.52-2.75 (2H, AB of ABX,  $J_{AB} = 17.1$  Hz, 3- $\underline{H}_2$ ), 3.58 (2H, d,  $J = 1.8$  Hz, Ar- $\underline{CH}_2$ ), 3.79 (3H, s, O- $\underline{CH}_3$ ), 4.53 (1H, m, 2- $\underline{H}$ ), 6.02 (1H, s, Ar- $\underline{H}$ ), 9.59 (1H, t,  $J = 1.7$  Hz,  $\underline{CH}_2$ -CHO), 12.13 (1H, s, Ar-OH)

$^{13}\text{C}$  N.M.R.: (50 MHz,  $\text{CDCl}_3$ )  $\delta$  20.6 (q), 36.9 (t), 43.1 (t),  
55.8 (q), 74.0 (d), 90.7 (d), 100.9 (s), 102.7 (s),  
160.9 (s), 162.7 (s), 165.5 (s), 191.1 (s),  
199.6 (d)

M.S.: m/z (%) 250 ( $\text{M}^+$ , 18), 222 (75), 221 (100),  
179 (82)

*5-hydroxy-6-(2-hydroxyethyl)-7-methoxy-2-methyl-4-chromanone (1)*

This was prepared in exactly the same way as was LL-D253 $\alpha$ -benzyl ether (52) (Method A), by borohydride reduction of 5-hydroxy-7-methoxy-2-methyl-6-(2-oxoethyl)-4-chromanone (60). Preparative t.l.c. of the crude extract, developing with acetone-chloroform (5:95), gave, on elution (ethyl acetate) of the band at  $R_f = 0.37$ , the required *alcohol* (1) as a white solid in 72% yield. Recrystallisation from ethyl acetate-hexane gave white needles, m.p. 136-137°C (sub.).

Found:  $\text{M}^+ = 252.0995$ .  $\text{C}_{13}\text{H}_{16}\text{O}_5$  requires  $\text{M} =$   
252.0998 nm

U.V.:  $\lambda_{\text{max}}^{\text{MeOH}}$  (log  $\epsilon$ ), 332 (3.41), 288 (4.22), 227 (sh,  
4.20), 215 (4.32) nm

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  3320 (br, m), 1648 (s), 1624 (s), 1577 (s),  
1297 (s)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (200 MHz,  $\text{CDCl}_3$ )  $\delta$  1.44 (3H, d,  $J = 6.3$  Hz,  $\text{CH}-\text{CH}_3$ ),  
1.94 (br s, ex,  $\text{CH}_2\text{OH}$ ), 2.48-2.72 (2H, AB of ABX,  
 $J_{\text{AB}} = 17.1$  Hz, 3- $\text{H}_2$ ), 2.81 (2H, t,  $J = 6.6$  Hz,  
 $\text{Ar}-\text{CH}_2$ ), 3.68 (2H, t,  $J = 6.6$  Hz,  $\text{CH}_2\text{OH}$ ),  
3.79 (3H, s,  $\text{O}-\text{CH}_3$ ), 4.39-4.58 (1H, m, 2- $\text{H}$ ),  
5.98 (1H, s,  $\text{Ar}-\text{H}$ ), 12.13 (1H, s, ex,  $\text{Ar}-\text{OH}$ )



$^{13}\text{C}$  N.M.R.: (90 MHz,  $\text{CDCl}_3$ )  $\delta$  20.7 (q), 25.4 (t), 43.3 (t),  
55.7 (q), 62.3 (t), 74.0 (d), 90.8 (d), 102.7  
(s), 106.6 (s), 160.8 (s), 161.9 (s), 165.6 (s),  
196.3 (s)

M.S.: m/z (%) 252 ( $\text{M}^+$ , 38), 222 (28), 221 (100),  
179 (57), 69 (12)

*5-hydroxy-8-(2,3-dihydroxypropyl)-7-methoxy-  
2-methyl-4-chromanone (59)*

To an ice-cold solution of osmium tetroxide (217 mg, 0.854 mmol) in dry pyridine (2 ml) stirred under a dry nitrogen atmosphere, was added an ice-cold solution of 8-allyl-5-hydroxy-7-methoxy-2-methyl-4-chromanone (57) (194 mg, 0.782 mmol) in dry pyridine (7 ml). The mixture rapidly darkened and was stirred at room temperature for 17 hours. Analytical t.l.c., developing with acetone-chloroform (5:95), showed that no alkene ( $R_f = 0.35$ ) remained so a solution of sodium metabisulphite (327 mg, 1.72 mmol) in water (3 ml) was added. A black precipitate formed from the opaque mixture to give a translucent solution. After a further 3 hours the total mixture was diluted with water (100 ml) and extracted with ethyl acetate (4 x 50 ml). The combined organic phases were washed with 2N HCl (4 x 30 ml), to remove the pyridine, dried ( $\text{MgSO}_4$ ) and reduced *in vacuo* to give *5-hydroxy-8-(2,3-dihydroxypropyl)-7-methoxy-2-methyl-4-chromanone (59)* (mixture of diastereoisomers) as a white crystalline solid (238 mg, 0.84 mmol, 107%) probably containing a little

acetic acid. Recrystallisation from ethyl acetate-hexane gave rosettes of white needles, m.p. 164-165.5°C.

Found:  $M^+ = 282.1087$ ,  $C_{14}H_{18}O_6$  requires  $M = 282.1103$ .

U.V.:  $\lambda_{\text{max}}^{\text{MeOH}}$  (log  $\epsilon$ ) 332 (3.43), 288 (4.16), 235 (sh, 3.98), 214 (4.30) nm

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  3380 (br, m), 1644 (s), 1626 (s), 1360 (m), 1122 (s)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (360 MHz,  $\text{CDCl}_3$ )  $\delta$  1.51 (3H, d,  $J = 6.3$  Hz,  $\text{CH}-\text{CH}_3$ ), 1.51-1.68 (br s, ex,  $\text{OH}$ ), 2.04-2.21 (br s, ex,  $\text{OH}$ ), 2.21-2.36 (br s, ex,  $\text{OH}$ ), 2.58-2.74 (2H, AB of ABX,  $3-\text{H}_2$ ), 2.72-2.88 (2H, m, 11 lines,  $\text{Ar}-\text{CH}_2$ ), 3.42-3.61 (2H, br m, sharpened by  $\text{D}_2\text{O}$  to 12 lines,  $\text{CH}_2-\text{OH}$ ), 3.81-3.90 (1H, br m, sharpened by  $\text{D}_2\text{O}$ ,  $-\text{CHOH}-$ ), 3.85 (3H, s,  $\text{O}-\text{CH}_3$ ), 4.47-4.60 (1H, m,  $2-\text{H}$ ), 6.09 (1H, s,  $\text{Ar}-\text{H}$ ), 12.17 (1H, s, ex,  $\text{Ar}-\text{OH}$ )

$^{13}\text{C}$  N.M.R.: (50 MHz,  $\text{CDCl}_3$ )  $\delta$  20.7 (q), 26.3 (t), 43.2 (t), 55.9 (q), 65.9 (t), 71.8 (d), 74.0 (d), 92.5 (d), 102.8 (s), 104.4 (s), 159.5 (s), 163.1 (s), 165.6 (s), 196.4 (s)

M.S.: m/z (%) 282 ( $M^+$ , 23), 251 (9), 222 (21), 221 (100), 209 (14), 179 (52).

*5-hydroxy-7-methoxy-2-methyl-8-(2-oxoethyl)-4-chromanone (61)*

5-hydroxy-8-(2,3-dihydroxypropyl)-7-methoxy-2-methyl-4-chromanone (59) (188 mg, 0.67 mmol), dissolved in methanol (10 ml), was added to a solution of sodium periodate (178 mg, 0.83 mmol) in water (5 ml) to form a cloudy suspension. The mixture was stirred at room temperature for 15 hours, diluted with water (150 ml) and extracted with ethyl acetate (4 x 50 ml). The organic extract was dried ( $\text{MgSO}_4$ ) and reduced *in vacuo* to give *5-hydroxy-7-methoxy-2-methyl-8-(2-oxoethyl)-4-chromanone (61)* as pale brown plates (157 mg, 0.63 mmol, 94%). Recrystallisation from deuteriochloroform gave large, clear, square prisms, m.p. 120.5-121.5°C.

Found:  $M^+ = 250.0836$ .  $\text{C}_{13}\text{H}_{14}\text{O}_5$  requires  $M = 250.0841$

U.V.:  $\lambda_{\text{max}}^{\text{CHCl}_3}$  (log  $\epsilon$ ) 330 (3.43), 289 (4.17), 241 (3.79) nm

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  1715 (s), 1647 (s), 1622 (s), 1592 (m), 1208 (s), 1115 (s)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (80 MHz,  $\text{CDCl}_3$ )  $\delta$  1.45 (3H, d,  $J = 6.3$  Hz,  $\text{CH}-\text{CH}_3$ ), 2.63 (2H, AB of ABX,  $3-\text{H}_2$ ), 3.56 (2H, d,  $J = 1.8$  Hz,  $\text{Ar}-\text{CH}_2$ ), 3.81 (3H, s,  $\text{O}-\text{CH}_3$ ), 4.26-4.76 (1H, m,  $2-\text{H}$ ), 6.08 (1H, s,  $\text{Ar}-\text{H}$ ), 9.57 (1H, t,  $J = 1.8$  Hz,  $-\text{CHO}$ ), 12.18 (1H, s, ex,  $\text{Ar}-\text{OH}$ )

$^{13}\text{C}$  N.M.R.: (50 MHz,  $\text{CDCl}_3$ )  $\delta$  20.6 (q), 37.4 (t), 43.1 (t), 55.9 (q), 74.1 (d), 92.3 (d), 99.9 (s), 102.7 (s), 159.8 (s), 163.8 (s), 165.5 (s), 196.4 (s), 199.5 (d)

M.S.: m/z (%), 250 ( $M^+$ , 16), 221 (72), 179 (100), 149 (12), 108 (14), 69 (32)

*5-hydroxy-8-(2-hydroxyethyl)-7-methoxy-2-methyl-4-chromanone (10)*

This was prepared in exactly the same way as was LL-D253 $\alpha$ -benzyl ether (52) (Method A), by borohydride reduction of 5-hydroxy-7-methoxy-2-methyl-8-(2-oxoethyl)-4-chromanone (61). Preparative t.l.c. of the crude extract developing with acetone-chloroform (5:95) gave, on elution (ethyl acetate) of the band at  $R_f = 0.40$ , the required *alcohol* (10) as a white crystalline solid in 57% yield. Recrystallisation from ethyl acetate-hexane gave white needles, m.p. 126-128.5°C. The relatively low yield probably reflects the presence of impurities in the aldehyde which was sensitive to handling.

Found:  $M^+ = 252.1001$ .  $C_{13}H_{16}O_5$  requires  $M = 252.0998$

U.V.:  $\lambda_{\max}^{\text{MeOH}}$  (log  $\epsilon$ ), 333 (3.48), 288 (4.21), 233 (sh, 4.03), 213 (4.34)

I.R.:  $\nu_{\max}^{\text{KBr}}$  3380 (br, m), 1648 (s), 1635 (sh, s), 1357 (m), 1120 (m)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (200 MHz,  $\text{CDCl}_3$ )  $\delta$ 1.45 (3H, d,  $J = 6.3$  Hz,  $\text{CH}-\text{CH}_3$ ), 1.95 (br s, ex,  $\text{CH}_2-\text{OH}$ ), 2.59 (2H, AB of ABX,  $J_{\text{AB}} = 17.1$  Hz, 3- $\text{H}_2$ ), 2.79 (2H, t,  $J = 6.8$  Hz, Ar- $\text{CH}_2$ ), 3.65 (2H, t,  $J = 6.8$  Hz,  $\text{CH}_2-\text{OH}$ ), 3.79 (3H, s, O- $\text{CH}_3$ ), 4.36-4.56 (1H, m, 2- $\text{H}$ ), 6.02 (1H, s, Ar- $\text{H}$ ), 12.12 (1H, s, ex, Ar-OH)

$^{13}\text{C}$  N.M.R.: (50 MHz,  $\text{CDCl}_3$ )  $\delta$ 20.6 (q), 25.7 (t), 43.1 (t),  
55.7 (q), 62.1 (t), 73.7 (d), 92.1 (d), 102.6 (s),  
105.1 (s), 159.5 (s), 162.8 (s), 165.7 (s), 196.5  
(s)

M.S.: m/z (%), 252 ( $\text{M}^+$ , 27), 221 (100), 179 (58), 149 (7),  
69 (11)

### 3.6.3 Biosynthetic and Related Experimental

#### *Production and Isolation of LL-D253 $\alpha$*

*Phoma pigmentivora* (QM 502) was stored in the dark at 4°C under liquid paraffin on slopes of corn-meal agar (Oxoid CM 103). A spore suspension in distilled water was used as the inoculum for a seed culture grown in 250 ml Erlenmeyer flasks on an orbital shaker. Each flask contained 75 ml of the medium:

Ammonium tartrate	0.2% w/v
Magnesium sulphate (heptahydrate)	0.05% w/v
Potassium chloride	0.05% w/v
Potassium orthophosphate	0.1% w/v
Ferrous sulphate (heptahydrate)	0.001% w/v
Glucose	5.0% w/v
Corn Steep Liquor	1.0% w/v
Distilled water	to 100%

(The pH was adjusted to 6.5 with 2N sodium hydroxide solution.)

After 3-4 days incubation at 26°C in constant light, the suspension of mycelial balls was used to inoculate further such flasks of the same medium which were in turn incubated under the same conditions for up to 12 days. The usual growth period was 5 days by which time the medium had turned appreciably darker in appearance.

The mycelium was removed by filtration (Whatman's No. 1) and washed with a little water. The medium and washings were acidified to approximately pH = 2 with dilute (2N) hydrochloric acid and extracted with ethyl acetate (3 or 4 times with one third of the liquor volume). After drying the extract ( $\text{MgSO}_4$ ), the solvent was removed *in vacuo* to leave a brown gum, the constituents of which were separated by preparative t.l.c., developing with methanol-chloroform (10:90). The band at approximately  $R_f = 0.5$  was eluted with methanol-ethyl acetate (5:95) to give, on evaporation of the solvent, LL-D253 $\alpha$  as a white crystalline solid. Yields were variable but were usually between 200 and 400 mg l<sup>-1</sup>. (Further purification by t.l.c. (if required) was effected using acetone-chloroform (20:80) with which two developments were necessary to give  $R_f = 0.5$ .) Recrystallisation from ethyl acetate-methanol afforded white needles, m.p. 192.5-193°C (lit.<sup>1</sup>, 188-188.5°C; lit.<sup>3</sup>, 191-193°C). Spectral data were identical to those of an authentic sample<sup>13</sup>.

Found: C, 61.69; H, 6.57.  $\text{C}_{13}\text{H}_{16}\text{O}_5$  requires C, 61.90; H, 6.39.

U.V.:  $\lambda_{\text{max}}^{\text{MeOH}}$  (log  $\epsilon$ ), 316 (sh, 3.68), 285 (4.23), 230 (sh, 4.13), 213 (4.28) nm

I.R.:  $\nu_{\text{max}}$  (KBr) 3160 (br, m), 1652 (m), 1595 (s), 1290 (m),  
1116 (m)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (300 MHz,  $\text{CDCl}_3$ )  $\delta$ 1.44 (3H, d,  $J = 6.3$  Hz,  $\text{CH}-\text{CH}_3$ ),  
2.49-2.63 (2H, AB of ABX, 3- $\text{H}_2$ ), 2.79-3.00 (2H, m,  
16 lines,  $\text{Ar}-\text{CH}_2$ ), 3.48 (s, OH), 3.84 (3H, s,  
 $\text{O}-\text{CH}_3$ ), 3.84-4.00 (2H, m,  $\text{CH}_2-\text{OH}$ ), 4.38-4.52  
(1H, m, 2- $\text{H}$ ), 6.13 (1H, s,  $\text{Ar}-\text{H}$ )

$^{13}\text{C}$  N.M.R.: (20 MHz,  $d_6$  DMSO)  $\delta$ 20.5 (q), 26.5 (t), 45.2 (t),  
55.4 (q), 60.1 (t), 73.3 (d), 92.7 (d), 104.5 (s),  
104.7 (s), 159.9 (s), 162.0 (s), 162.2 (s),  
188.5 (s)

M.S.:  $m/z$  (%) 252 (53), 221 (83), 179 (100)

*LL-D253 $\alpha$  diacetate (17)*

LL-D253 $\alpha$  (16) (250 mg, 0.99 mmol) was dissolved in  
redistilled acetic anhydride (5 ml); pyridine (5 drops) was  
added, and the mixture was heated under gentle reflux for  
10 minutes. Moisture was carefully excluded. The solvents  
were removed *in vacuo*, the final traces being azeotroped  
with chloroform and carbon tetrachloride, to leave LL-D253 $\alpha$   
diacetate (17) (336 mg, 1.00 mmol, 100%), as a white  
crystalline solid, m.p. 120.5-122.5°C. Recrystallisation from  
ethyl acetate-hexane yielded small white prisms, m.p.  
122.5-124.5°C (lit.<sup>1</sup>, 121-122°C). (If the starting material  
was not completely pure, the diacetate could be purified  
by preparative t.l.c., developing with methanol-chloroform  
(2:98),  $R_f = 0.7$ , and eluting with ethyl acetate.)

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  1767 (s), 1734 (s), 1689 (s), 1591 (s)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (360 MHz,  $\text{CDCl}_3$ )  $\delta$ 1.48 (3H, d,  $J = 6.3$  Hz,  $\text{CH}-\text{CH}_3$ ),  
2.01 (3H, s,  $\text{CH}_2-\text{OCO}-\text{CH}_3$ ), 2.34 (3H, s,  $\text{Ar}-\text{OCO}-\text{CH}_3$ ),  
2.57-2.67 (2H, AB of ABX, 3- $\text{H}_2$ ), 2.82 (2H, t,  
 $J = 7.0$  Hz,  $\text{Ar}-\text{CH}_2$ ), 3.85 (3H, s,  $\text{O}-\text{CH}_3$ ), 4.14 (2H,  
t,  $J = 7.0$  Hz,  $\text{CH}_2-\text{OAc}$ ), 4.50-4.61 (1H, m, 2- $\text{H}$ ),  
6.26 (1H, s,  $\text{Ar}-\text{H}$ )

$^{13}\text{C}$  N.M.R.: (50 MHz,  $\text{CDCl}_3$ )  $\delta$ 20.0 (q), 20.2 (q), 20.3 (q),  
22.7 (t), 44.9 (t), 55.5 (q), 62.2 (t), 73.6 (d),  
98.5 (d), 108.8 (s), 110.2 (s), 154.5 (s), 159.3 (s),  
161.5 (s), 168.0 (s), 170.2 (s), 189.4 (s)

*Growth-production study of LL-D253 $\alpha$*

*Phoma pigmentivora* was grown as described. At intervals of approximately 12 hours, two 250 ml Erlenmeyer flasks were removed from culture and worked up.

Results:

Time after Inoculation/hours	Mycelial Dry Weight/g $\text{l}^{-1}$	Extract Weight/g $\text{l}^{-1}$	LL-D253 $\alpha$ Weight/mg $\text{l}^{-1}$
8	6.3	0.27	
24.5	2.7	0.29	20
36	3.4	0.33	40
48	5.6	0.39	133
57	5.0	0.33	53
72.5	5.8	0.37	66
83	5.6	0.48	133
96	6.4	0.41	100
122	4.5	0.37	87
132	8.7	0.55	207
151	10.4	0.51	247
170	11.9	0.56	373



Results (contd.)

Time after Inoculation/hours	Mycelial Dry Weight/g l <sup>-1</sup>	Extract Weight/g l <sup>-1</sup>	LL-D253α Weight/mg l <sup>-1</sup>
180	11.1	0.62	300
193	11.7	0.80	333
204	11.5	0.65	307
217.5	12.3	0.65	213
224.5	13.0		
241.5	12.4	0.87	333
250.5		0.57	320
264	12.8	0.88	447
276	11.9	0.82	360

*Incorporation of sodium [2-<sup>14</sup>C]acetate into LL-D253α*

*Phoma pigmentivora* was grown as described in 24 flasks. Twelve hours after inoculation, a sterile solution of sodium [2-<sup>14</sup>C]acetate (500 mg, 10 μCi, Sp.Act. =  $3.64 \times 10^6$  dpm mmol<sup>-1</sup>) in distilled water (12 ml) was distributed evenly by syringe among 6 flasks. Parallel experiments, feeding at 57 hours and 105 hours, were conducted concurrently. Six flasks were retained as controls. All flasks were incubated for a total of 174 hours. Isolation and purification of LL-D253α followed.

Results:

Experiment	Yield of LL-D253 /mg l <sup>-1</sup>	Specific Activity/ dpm mmol <sup>-1</sup>	Dilution
Control	176		
12 hour feed	362	$4.3 \times 10^5$	8.5
57 hour feed	311	$1.3 \times 10^6$	2.8
105 hour feed	218	$4.5 \times 10^5$	8.1

*Incorporation of sodium [1-<sup>13</sup>C]-, [2-<sup>13</sup>C]- and [1,2-<sup>13</sup>C<sub>2</sub>]acetates into LL-D253α (No dilution of labelled precursor)*

*Phoma pigmentivora* was grown as described in 8 flasks. 57.5 hours after inoculation, a sterile solution of sodium [1-<sup>13</sup>C]acetate (200 mg, 92.6 atom % <sup>13</sup>C) in distilled water (8 ml) was distributed evenly by syringe between 2 flasks. Parallel experiments, feeding with sodium [2-<sup>13</sup>C]acetate (200 mg, 97.5 atom % <sup>13</sup>C) and sodium [1,2-<sup>13</sup>C<sub>2</sub>]acetate (200 mg, 91.6, 91.5 atom % <sup>13</sup>C) again at 57.5 hours, were conducted concurrently. All the flasks were incubated for a total of 126 hours after which the LL-D253α was isolated.

Results:

Experiment	Yield of LL-D253α/mg l <sup>-1</sup>
Control	100
[1- <sup>13</sup> C]acetate feed	107
[2- <sup>13</sup> C]acetate feed	93
[1,2- <sup>13</sup> C <sub>2</sub> ]acetate feed	93

*Incorporation of sodium [1-<sup>13</sup>C]-, [2-<sup>13</sup>C]- and [1,2-<sup>13</sup>C<sub>2</sub>]acetates into LL-D253α (with dilution of labelled precursor)*

*Phoma pigmentivora* was grown as described in 25 flasks. 58 hours after inoculation, a sterile solution of sodium [1-<sup>13</sup>C]acetate (200 mg at 91.7 atom % <sup>13</sup>C; 400 mgs at natural abundance) in distilled water (20 ml) was distributed evenly by syringe among 4 flasks. Parallel experiments, feeding with sodium [2-<sup>13</sup>C]acetate (200 mg at 90.6 atom % <sup>13</sup>C; 400 mgs at natural abundance) and sodium [1,2-<sup>13</sup>C<sub>2</sub>]acetate (200 mg at 91.8, 90.6 atom % <sup>13</sup>C; 400 mg at natural abundance), were conducted concurrently. All the flasks including 13 control flasks were incubated for a total of 126 hours. LL-D253α was isolated as usual.

Results:

Experiment	Yield of LL-D253α/mg l <sup>-1</sup>
Control	317
[1- <sup>13</sup> C]acetate feed	437
[2- <sup>13</sup> C]acetate feed	413
[1,2- <sup>13</sup> C <sub>2</sub> ]acetate feed	453

*Incorporation of sodium [1-<sup>14</sup>C]acetate, sodium [1-<sup>14</sup>C]-glycollate, [U-<sup>14</sup>C]oxalic acid and sodium[1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate into LL-D253α*

*Phoma pigmentivora* was grown as described in 20 flasks. 71 hours after inoculation the following compounds were administered as sterile solutions in distilled water.

- (1) Sodium [1-<sup>14</sup>C]acetate (58.2 mCi mmol<sup>-1</sup>, 0.08 μCi ml<sup>-1</sup>);  
2 ml to each of 5 flasks.
- (2) Sodium [1-<sup>14</sup>C]glycollate (5 mCi mmol<sup>-1</sup>, 1 μCi ml<sup>-1</sup>);  
2 ml to each of 5 flasks.
- (3) [U-<sup>14</sup>C]oxalic acid (98 mCi mmol<sup>-1</sup>, 1 μCi ml<sup>-1</sup>);  
2 ml to each of 5 flasks.
- (4) Sodium [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate (150 mg at 90 atom % <sup>13</sup>C, 81 atom % <sup>18</sup>O<sub>2</sub>, 18 atom % <sup>18</sup>O; 300 mg at natural abundance together in 9 ml); 3 ml to each of 3 flasks.

The remaining 2 flasks were retained as controls and 20 flasks were incubated for a total of 148 hours. LL-D253α was then isolated as usual.

### Results:

Experiment	Yield/ mg l <sup>-1</sup>	Sp. Act./ dpm mmol <sup>-1</sup>	Dilution	Percentage Incorporation
Control	267			
(1)	189	4.6 x 10 <sup>5</sup>	2.8 x 10 <sup>5</sup>	7.2
(2)	368	5.7 x 10 <sup>4</sup>	2.0 x 10 <sup>5</sup>	0.14
(3)	363	1.7 x 10 <sup>6</sup>	1.3 x 10 <sup>5</sup>	4.2
(4)	382			

The percentage incorporation figures are based on the quantities of LL-D253 $\alpha$  initially isolated, before purification to constant activity, and on the final specific activities.

*Attempted incorporation of (3R)-[5-<sup>14</sup>C]mevalonate into LL-D253 $\alpha$*

To a benzene solution (0.5 ml, 50  $\mu$ Ci ml<sup>-1</sup>) of (3R)-[5-<sup>14</sup>C]mevalonic acid lactone (57 mCi mmol<sup>-1</sup>) was added aqueous potassium hydroxide (1 ml, 0.1 N). The mixture was stirred at 50-60°C for one hour while the benzene was removed by a stream of nitrogen; the aqueous residue was then diluted to 10 ml with distilled water. This solution of potassium mevalonate was sterilised and distributed evenly by syringe among 5 flasks of a 48 hour culture of *Phoma pigmentivora*. Also administered, also at 48 hours, to each flask was a sterile solution (2 ml) of sodium acetate (57 mg ml<sup>-1</sup>) in distilled water. 37 further flasks were each fed only the sodium acetate solution (2 ml) at this time. Six control flasks were fed nothing. All the flasks were incubated for a total of 166 hours after which time the LL-D253 $\alpha$  was isolated as usual.

#### Results:

Experiment	Yield/mg l <sup>-1</sup>
Control	271
Fed acetate	273
Fed acetate and (3R)-[5- <sup>14</sup> C]mevalonate	357

Although highly active after the initial chromatographic purification, after two recrystallisations the mevalonate-labelled sample had a specific activity of only *ca.*  $1 \times 10^4$  dpm  $\text{mmol}^{-1}$  (*i.e.* just above background). This corresponds to a dilution value of *ca.*  $1 \times 10^7$ . Based on the quantity of LL-D253 $\alpha$  initially isolated (134 mg) and on the final activity, the percentage incorporation was *ca.* 0.01%.

*Incorporation of sodium [ $1\text{-}^{13}\text{C}$ ,  $^2\text{H}_3$ ]acetate into LL-D253 $\alpha$*

To a 60 hour culture of *Phoma pigmentivora* was fed a sterile solution of sodium [ $1\text{-}^{13}\text{C}$ ,  $^2\text{H}_3$ ]acetate (250 mg at 90 atom %  $^{13}\text{C}$ , 98 atom %  $^2\text{H}$ ; 500 mg at natural abundance) in distilled water (20 ml), distributed evenly by syringe among 5 flasks. After a total incubation time of 144 hours, LL-D253 $\alpha$  (55 mg) was isolated. The yield was low due to an accident during work-up.

*Demethylation of LL-D253 $\alpha$  by boron trichloride*

LL-D253 $\alpha$  (50 mg, 0.198 mmol) was suspended in dry methylene chloride (15 ml) and the mixture was chilled to  $-50^\circ\text{C}$ . Boron trichloride (0.5 ml, 0.7 g, 6 mmol), also chilled to  $-50^\circ\text{C}$ , was added and the flask was immediately sealed. The LL-D253 $\alpha$  dissolved instantly and the solution became yellow. After it had stood at room temperature for 30 minutes, the flask was rechilled to  $-50^\circ\text{C}$  and opened. The excess boron trichloride was removed by attaching the

flask to the rotary evaporator (without heating) for 15 minutes: the residual solution was diluted (to 50 ml) with further methylene chloride, washed with water (4 x 20 ml), dried ( $\text{MgSO}_4$ ) and reduced *in vacuo* to yield 5,7-dihydroxy-8-(2-hydroxyethyl)-2-methyl-4-chromanone (40 mg, 0.168 mmol, 85%) as a white crystalline solid. Recrystallisation from methanol-ethyl acetate gave white triangular plates, m.p. 171-173°C (sub.).

Found:  $M^+ = 238.0870$ .  $\text{C}_{12}\text{H}_{14}\text{O}_5$  requires  $M = 238.0841$

U.V.:  $\lambda_{\text{max}}^{\text{MeOH}}$  (log  $\epsilon$ ), 329 (3.48), 290 (4.21), 213 (4.26) nm

I.R.:  $\nu_{\text{max}}^{\text{KBr}}$  3310 (m, br), 1642 (s), 1630 (s), 1613 (s), 1348 (m), 1305 (m), 1162 (m)  $\text{cm}^{-1}$

$^1\text{H}$  N.M.R.: (200 MHz,  $(\text{CD}_3)_2\text{CO}$ )  $\delta$  1.48 (3H, d,  $J = 6.3$  Hz,  $\text{CH-CH}_3$ ), 2.56-2.79 (2H, AB of ABX,  $J_{\text{AB}} = 17.2$  Hz, 3- $\text{H}_2$ ), 2.84 (2H, 't', sharpened by  $\text{D}_2\text{O}$ ,  $J = 7.4$  Hz, Ar- $\text{CH}_2$ ), 3.71 (2H, 't', sharpened by  $\text{D}_2\text{O}$ ,  $J = 7.4$  Hz,  $\text{CH}_2\text{-OH}$ ), 4.50-4.68 (1H, m, 2- $\text{H}$ ), 5.96 (1H, s, Ar- $\text{H}$ ), 12.16 (1H, s, ex, 5-OH)

$^{13}\text{C}$  N.M.R.: (50 MHz,  $(\text{CD}_3)_2\text{CO}$ )  $\delta$  19.3 (q), 25.1 (t), 41.9 (t), 61.0 (t), 73.3 (d), 95.3 (d), 101.5 (s), 104.4 (s), 159.8 (s), 161.7 (s), 164.3 (s), 196.1 (s)

M.S.: m/z (%), 238 ( $M^+$ , 38), 207 (100), 165 (76), 69 (14).

*Demethylation of oxalate-labelled LL-D253α*

LL-D253α (88 mg, 0.349 mmol,  $1.7 \times 10^6$  dpm mmol<sup>-1</sup>) labelled from [U-<sup>14</sup>C]oxalic acid was demethylated by boron trichloride as described above to yield 5,7-dihydroxy-8-(2-hydroxyethyl)-2-methyl-4-chromanone (63 mg, 0.265 mmol, 76%). This was recrystallised to constant activity:  $3.2 \times 10^4$  dpm mmol<sup>-1</sup>. Thus 98% of the activity had been lost.

*Incorporation of sodium [<sup>2</sup>H<sub>3</sub>]acetate into LL-D253α*

To a 57.5 hours culture of *Phoma pigmentivora* was fed a sterile solution of sodium [<sup>2</sup>H<sub>3</sub>]acetate (1.00 g, 99 atom % <sup>2</sup>H) in distilled water (20 ml), distributed evenly among 10 flasks. After 264 hours of incubation, LL-D253α (283 mg, 377 mg l<sup>-1</sup>) was isolated.

*Preparation of generally deuterated LL-D253α*

*Phoma pigmentivora* was grown as previously described in 7 flasks. In 5 of these, 5% of the water had been substituted by D<sub>2</sub>O. The flasks were incubated for 192 hours and the LL-D253α was isolated as usual.

Results:

Experiment	Yield/mg l <sup>-1</sup>
Control	273
In D <sub>2</sub> O	325



*Incorporation of  $^{18}\text{O}_2$  into LL-D253 $\alpha$*

*Phoma pigmentivora* was grown, as previously described, in four flasks. Two were fitted with foam rubber bungs as usual and retained as controls. The other two were attached to the "constant pressure" apparatus (Appendix ) for measuring the uptake of oxygen and grown under an atmosphere of  $^{18}\text{O}_2$ - $^{14}\text{N}_2$  (20:80). After 123 hours growth both pairs of flasks were worked up and LL-D253 $\alpha$  was isolated as usual.

Results:

Time after inoculation/ hours	Volume $^{18}\text{O}_2$ consumed/ litres
29	0.20
57	0.58
75.5	0.86
81.5	0.95
98	1.31
104	1.40
123	1.66

Yield of LL-D253 $\alpha$ :

Control	240 mg l <sup>-1</sup>
Experimental	253 mg l <sup>-1</sup>

*Preparation of [1',3-<sup>2</sup>H<sub>4</sub>]LL-D253α-benzyl ether*

7-benzyloxy-5-methoxy-2-methyl-8-(2-oxoethyl)-4-chromanone (51) (47 mg, 0.138 mmol) was dissolved in CD<sub>3</sub>OD (0.5 ml) in a 5 mm n.m.r. tube. The 60 MHz <sup>1</sup>H n.m.r. spectrum was complex and, in particular, the aldehyde signal which, in CDCl<sub>3</sub>, occurs at 9.60 p.p.m. was very small: this suggested that the substrate was present largely in hemiacetal and/or acetal forms.

The sample was left at room temperature for 60 hours after which another <sup>1</sup>H n.m.r. spectrum was obtained. No change was indicated so two drops of NaOCD<sub>3</sub> solution (Na, 9 mg in CD<sub>3</sub>OD, 0.25 ml) were added. The clear solution in the n.m.r. tube rapidly adopted a yellow colour. After 40 minutes at room temperature, a further <sup>1</sup>H n.m.r. spectrum indicated the absence of any signals attributable to methylene groups adjacent to carbonyls. The aldehyde signal was now completely absent.

The solution was taken up in ethyl acetate (20 ml), washed with water (3 x 5 ml), dried (MgSO<sub>4</sub>) and reduced *in vacuo* to give [3-<sup>2</sup>H<sub>2</sub>]-7-benzyloxy-5-methoxy-2-methyl-8-([1-<sup>2</sup>H<sub>2</sub>]-2-oxoethyl)-4-chromanone (88) as a pale brown gum (40 mg, 0.116 mmol, 84%). A <sup>1</sup>H n.m.r. spectrum (60 MHz, CDCl<sub>3</sub>) showed that the signals, at 2.60 p.p.m. and 3.67 p.p.m. in the unlabelled material, due, respectively, to the 3-methylene and benzylic methylene groups, were absent. The aldehyde signal at 9.60 p.p.m., previously a triplet, had been replaced by a singlet.

This material was not purified but was reduced using sodium borohydride as described in the synthetic details (Section 3.6.2). The resulting  $[1',3\text{-}^2\text{H}_4]\text{LL-D253}\alpha\text{-benzyl ether}$  (89) was cleaned up by t.l.c., also as previously described, to give a clear gum (29 mg, 0.084 mmol, 72%).

A  $^1\text{H}$  n.m.r. spectrum (200 MHz,  $\text{CDCl}_3$ ) showed only vestigial signals at 2.68 p.p.m. and 2.95 p.p.m. Integration indicated that the compound was about 90% deuterated at the positions corresponding to these signals. The resonances at 3.77 p.p.m. and around 4.5 p.p.m., a triplet and a multiplet respectively in the spectrum of the unlabelled compound, showed here as a singlet and a quartet. A  $^2\text{H}$  n.m.r. spectrum (55.3 MHz,  $\text{CHCl}_3$ ) displayed two broad singlets at 2.56 and 2.91 p.p.m. Mass spectrometry gave a predominant parent-ion peak at  $m/z = 346$  ( $M + 4$ ).

*Hydrogenolysis of  $[1',3\text{-}^2\text{H}_4]\text{LL-D253}\alpha\text{-benzyl ether}$*

This was carried out in the same manner as previously described in the synthetic details (Section 3.6.2). The benzyl ether (89) (24 mg, 0.069 mmol) thus yielded  $[1',3\text{-}^2\text{H}_4]\text{LL-D253}\alpha$  (90) (11 mg, 0.043 mmol, 62%). The  $^1\text{H}$  n.m.r. spectrum (200 MHz,  $\text{CD}_3\text{OD}$ ) was compared with one of natural LL-D253 $\alpha$  (80 MHz,  $\text{CD}_3\text{OD}$ ). Two signals in the latter spectrum: the AB of ABX pattern between 2.58 and 2.68 p.p.m. and the triplet centered at 2.92 p.p.m., assigned, respectively, to the 3-methylene and benzylic methylene groups, were only present to the extent of 10% of their natural intensity in the spectrum of the labelled material.

The multiplet at around 4.6 p.p.m. and the triplet at 3.72 p.p.m. were simplified to a quartet and a singlet in the spectrum of the labelled compound.

*Attempted acid-catalysed rearrangement of [1',3-<sup>2</sup>H<sub>4</sub>]-LL-D253α*

[1',3-<sup>2</sup>H<sub>4</sub>]LL-D253α (90) (11 mg, 0.043 mmol) was heated under reflux in dry benzene (10 ml) with *para*-toluene-sulphonic acid (1 mg) for 16 hours. Precautions were taken to exclude moisture. The benzene was removed *in vacuo* and the resulting brown gum was examined by <sup>1</sup>H n.m.r. spectroscopy (80 MHz, CD<sub>3</sub>OD). The signal at 2.92 p.p.m. was still absent (see above) but that between 2.58 and 2.68 p.p.m. was present to some extent, although still reduced relative to the corresponding signal in a spectrum of natural LL-D253α. A <sup>2</sup>H n.m.r. spectrum (55.3 MHz, CH<sub>3</sub>OH) of the product confirmed that deuterium was still present at the 3-methylene position (2.6 p.p.m.) but to only about 40% of the level at the benzylic methylene group (2.9 p.p.m.).

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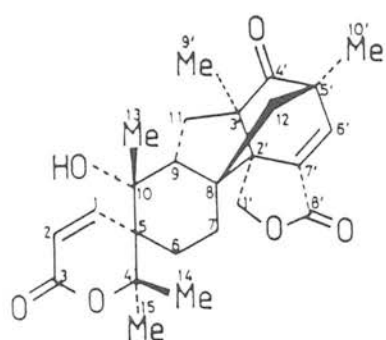
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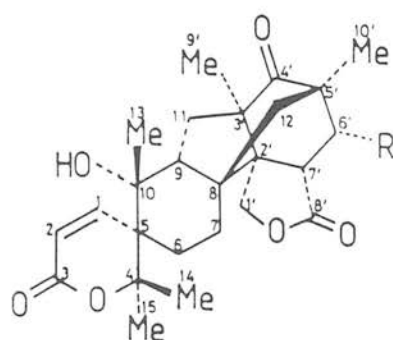
Chapter 4

Biosynthetic Studies on Some *Aspergillus*

Triprenyl-Phenols

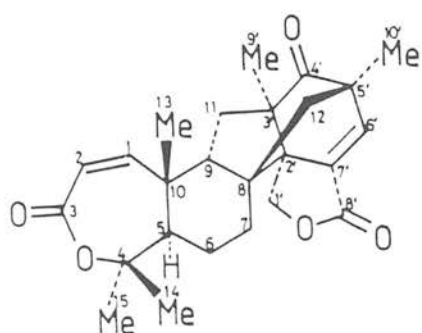


(1)

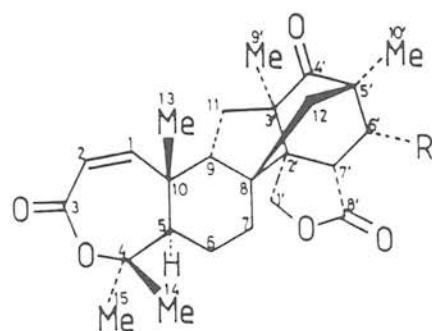


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(3) R = H

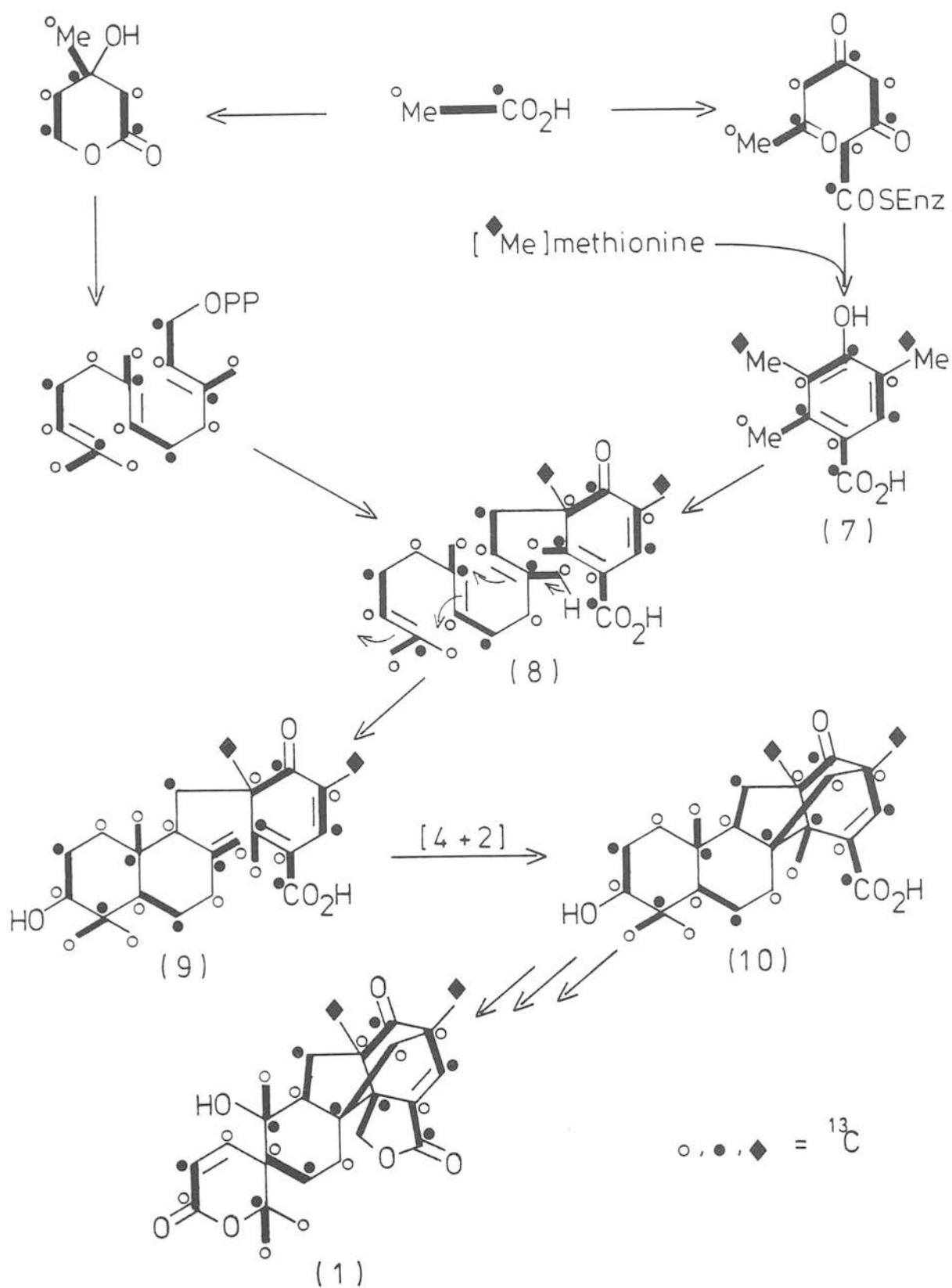


(5)



(4) R = OH

(6) R = H



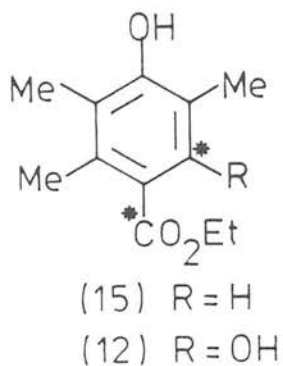
Scheme 1 Biosynthesis of andibenin B

4      Biosynthetic Studies on Some *Aspergillus*  
         Triprenyl-Phenols

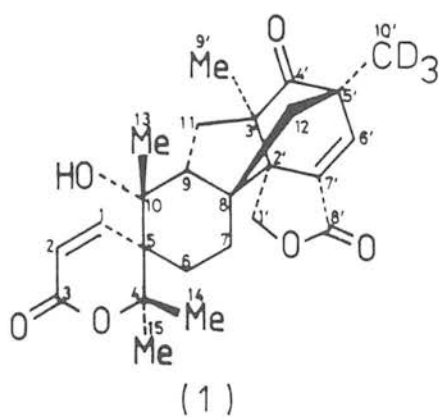
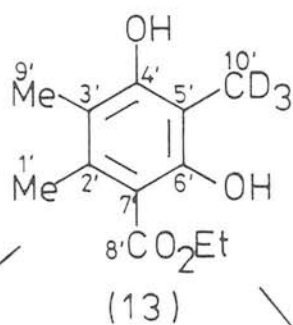
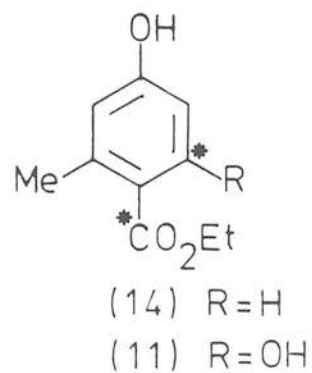
4.1    Background Information

The X-ray structure<sup>1,3</sup> and absolute stereochemistry of andibenin B (1), isolated from *Aspergillus variegator*, were first reported in 1976 and a polyisoprenoid biogenesis was suggested<sup>1</sup>. Two related metabolites, named dihydro-andibenin and deoxyandibenin, were also isolated<sup>1</sup>, but were subsequently renamed andilesin A (4) and andilesin B (5) after X-ray crystallography<sup>2,3</sup> established the skeletal difference between them and andibenin B, and spectroscopic<sup>3,4</sup> and chemical<sup>4</sup> study revealed their structural relationship. Additional metabolites, andilesin C<sup>2,3,4</sup> (6), and andibenins<sup>4</sup> A (2) and C (3), were also isolated and characterised.

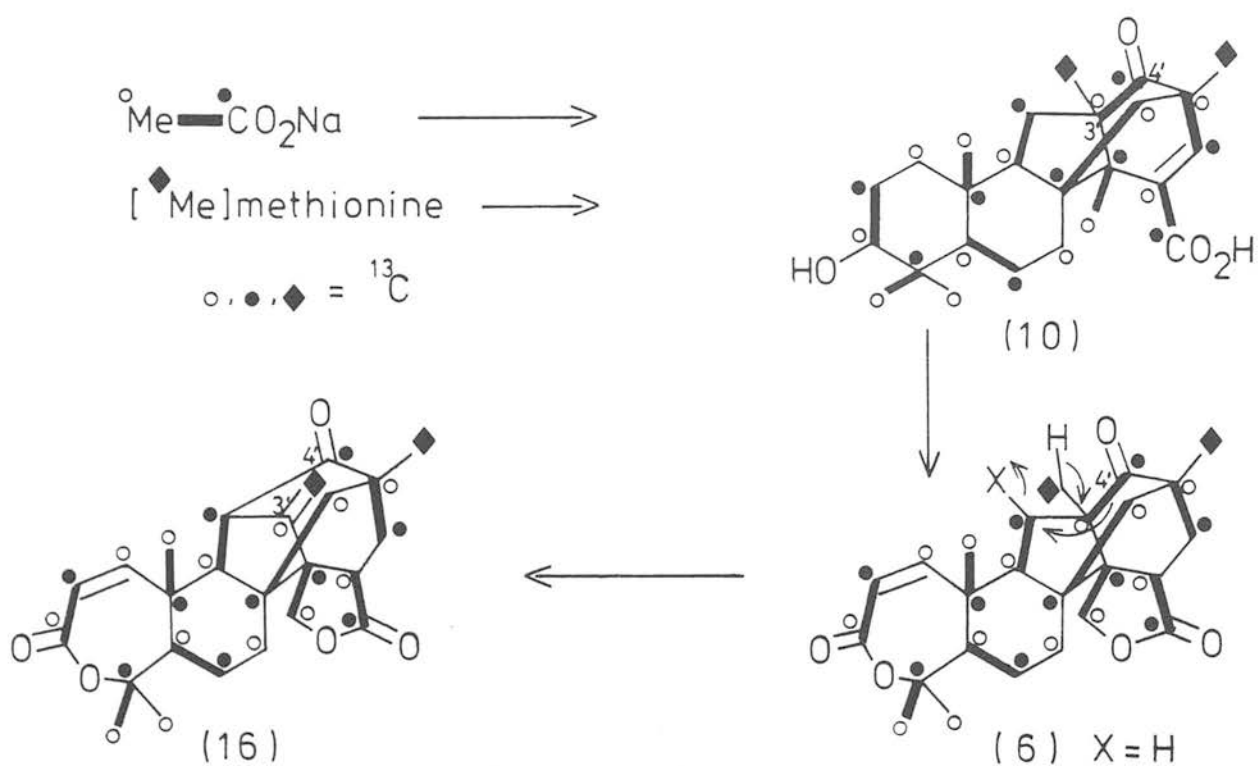
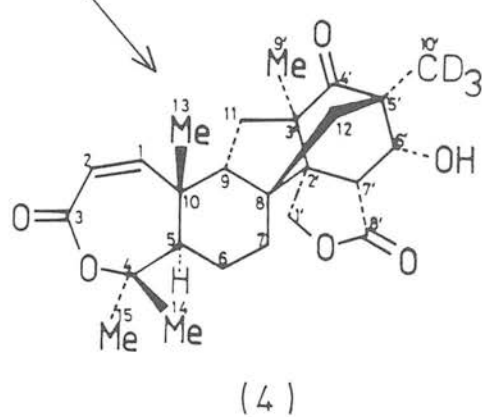
Biosynthetic investigation<sup>5</sup> of andibenin B (1), in which [1-<sup>13</sup>C]-, [2-<sup>13</sup>C]- and [1,2-<sup>13</sup>C<sub>2</sub>]acetates and [Me-<sup>13</sup>C]methionine were fed to *A. variegator*, however, showed a labelling pattern (Scheme 1) inconsistent with a sesterterpenoid origin. A mixed tetraketide-sesquiterpene biogenesis was indicated and a route was proposed (Scheme 1) whereby alkylation of a phenol (7) with farnesyl pyrophosphate gave the intermediate species (8): after cyclisation to the triene (9), a [4 + 2] cycloaddition could generate the required carbon skeleton (10).



\* =  $^{14}\text{C}$



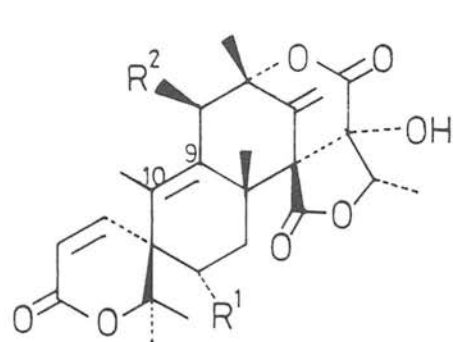
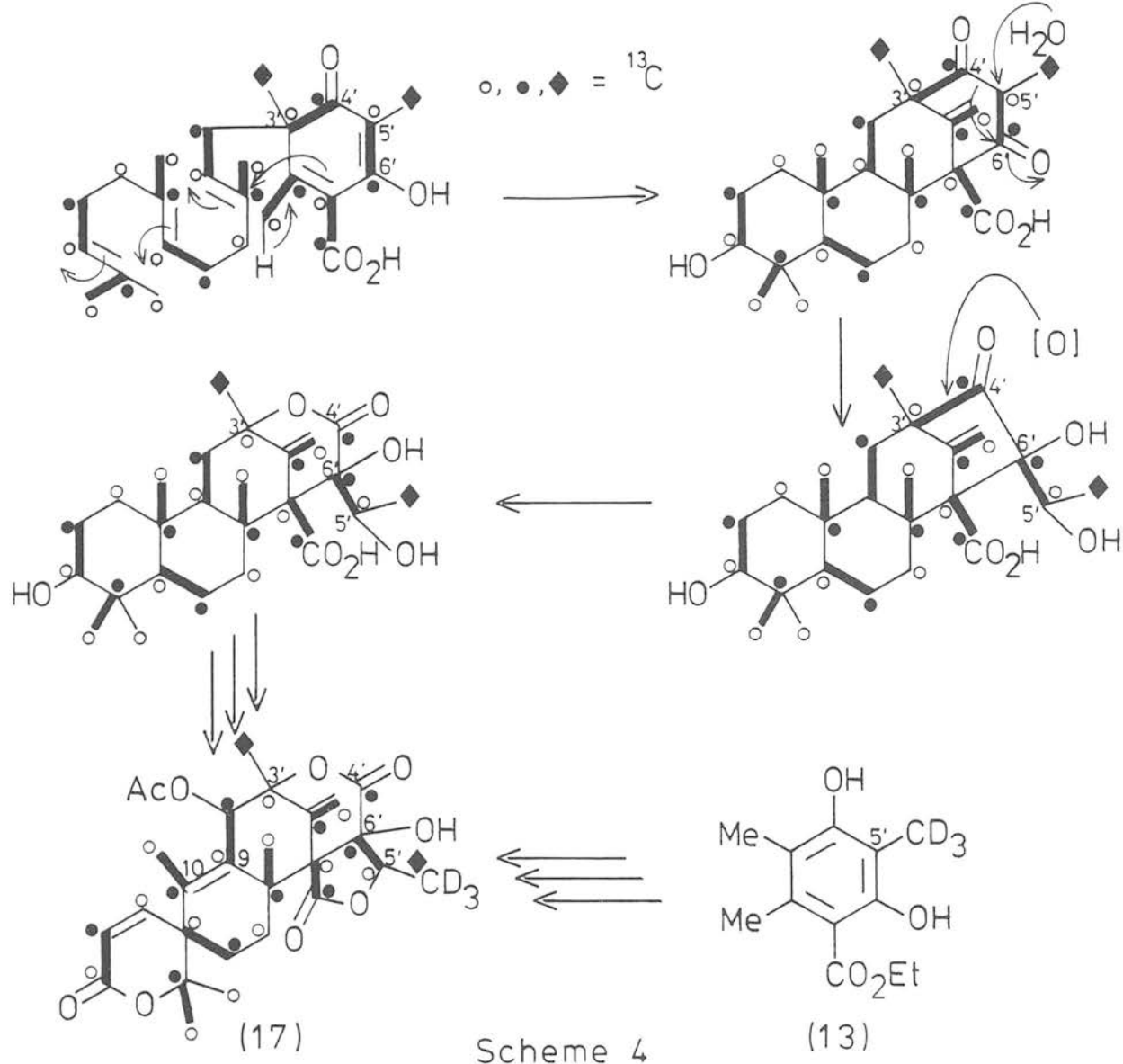
Scheme 2



Although several fungal triprenyl-phenols had been previously described<sup>6</sup>, the degree of skeletal modification required to form the andibenins was unprecedented. Structural analysis<sup>4</sup> suggested that the andilesins might be precursors to the andibenins, and that the most likely biosynthetic sequence within each series would be A to B to C by a sequence of dehydration and reduction. This, and the general observation<sup>7</sup> (see Section 1.3) that the 3-keto oxygen of polyketide chains cyclised at the 2-methylene position is always retained in the stabilised polyketide, in turn implied that an orsellinate might be the true tetraketide intermediate, giving initially andilesin A (4).

A number of orsellinates (11), (12), (13) and deoxyorsellinates (14), (15) were synthesised<sup>8</sup> and fed to *A. variegata*<sup>9</sup>. Both 3,5-dimethylorsellinate (12) and the deoxy-analogue (15) were well incorporated into andibenin B although the former was utilised some 2.5 times more efficiently. The specificity of labelling was established by feeding the deuterated orsellinate (13): the andibenin B and andilesin A subsequently isolated both showed a single resonance in their <sup>2</sup>H n.m.r. spectra. In each case this corresponded to the 10'-methyl group (Scheme 2). In contrast, the unmethylated compounds (11), (14) did not label andibenin B to any significant extent, establishing that the methyl groups previously shown<sup>5</sup> to be methionine-derived in andibenin were incorporated into the precursor tetraketide before aromatisation.

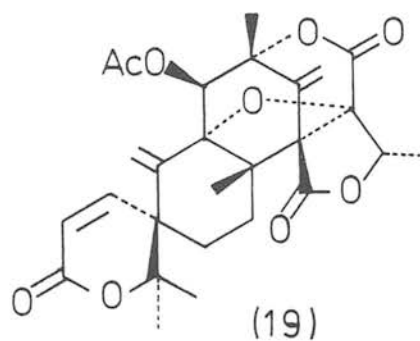
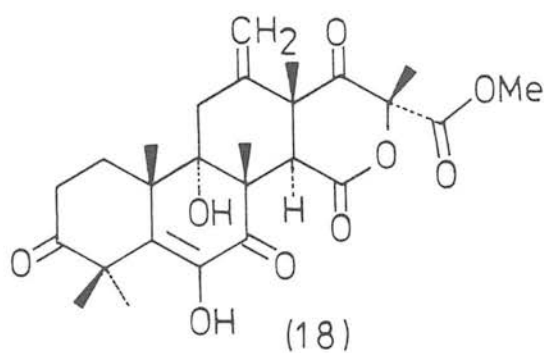




(17)  $\text{R}^1 = \text{H}$ ,  $\text{R}^2 = \text{OAc}$

(20)  $\text{R}^1 = \text{H}$ ,  $\text{R}^2 = \text{OH}$

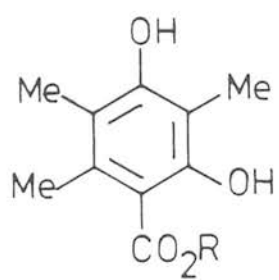
(21)  $\text{R}^1 = \text{OAc}$ ,  $\text{R}^2 = \text{H}$



Incorporation<sup>10</sup> of  $^{13}\text{C}$ -labelled acetates and  $[\text{Me-}^{13}\text{C}]$ -methionine into anditomin<sup>11</sup> (16), a further *A. variegator* metabolite with obvious structural similarities to the andilesins, showed that the skeletal differences and carbon-labelling pattern could be accounted for by a cleavage of the tetraketide ring (Scheme 3). Andilesin C (6) was a plausible intermediate.

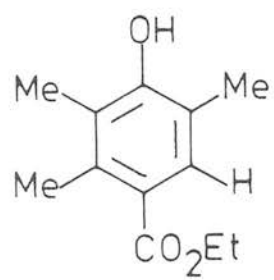
Possible evidence that the general pathway was not restricted to *A. variegator* arose with the isolation of austin<sup>12</sup> (17) from *Aspergillus ustus* and terretonin<sup>13</sup> (18) from *Aspergillus terreus*. X-ray crystallography provided the structures and in each case a polyisoprenoid origin was initially proposed. However, incorporation of  $^{13}\text{C}$ -labelled acetates and methionine<sup>14</sup> and subsequently 3,5-dimethyl-orsellinates<sup>15</sup> (12), (13) into austin (Scheme 4) showed that it was indeed formed by the meroterpenoid<sup>16</sup> route of the *A. variegator* metabolites, albeit with a remarkable degree of modification to the tetraketide portion. Interestingly, in this case the 2-deoxyorsellinate (15) was not significantly incorporated<sup>15</sup>.

Two new related compounds, dehydroaustin (19) and austinol (20), initially isolated from *A. ustus*, were later obtained<sup>17</sup> from, respectively, an *A. variegator* mutant and *Penicillium diversum*; this latter species also produced iso-austin (21). Another group reported<sup>18</sup> further austin-type variants, this time from *Emericella dentata*, further widening the range of species in which the pathway could be implicated.

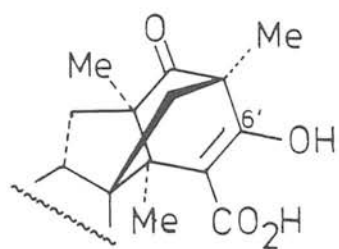


(12) R = Et

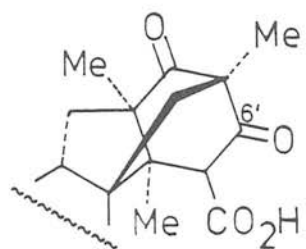
(22) R = H



(15)



(23)



(24)

## 4.2 $^{18}\text{O}$ -Labelling of Andibenin B and Andilesin A

### 4.2.1 Object of Experiments

The observation<sup>9</sup> that both ethyl 3,5-dimethyl-orsellinate (12) and its deoxy-analogue (15) were effective precursors of andibenin B (1) raises questions about the biosynthetic origin of the 6'-hydroxyl group of andibenin A (2) and andilesin A (4). This is in the right position to be acetate-derived *via* the orsellinate (22). However, the postulated [4 + 2] cycloaddition would then yield the enol moiety (23) or its ketone tautomer (24), and none of the andibenins or andilesins has either structural feature. If then, the cycloaddition occurs as suggested in Scheme 1 to give initially andibenin B and andilesin B (5), the 6'-hydroxyl group of the A compounds (2), (4) must originate from either the aqueous medium or the atmosphere.

The formation of the unusual spiro-lactone ring system is also interesting. All the andibenins include a hydroxyl group of unknown origin at C-10, whereas austin (17), which possesses the same ring system, exhibits a double-bond between C-9 and C-10. Reasonable mechanisms for the necessary rearrangement can be envisaged, distinguishable by the source of the oxygen atom of the C-10 hydroxy group of andibenin B. For these reasons, the object of the experiments to be described here was to investigate the biosynthetic origin of the oxygen atoms of andibenin B and andilesin A using  $^{18}\text{O}$ -labelling.

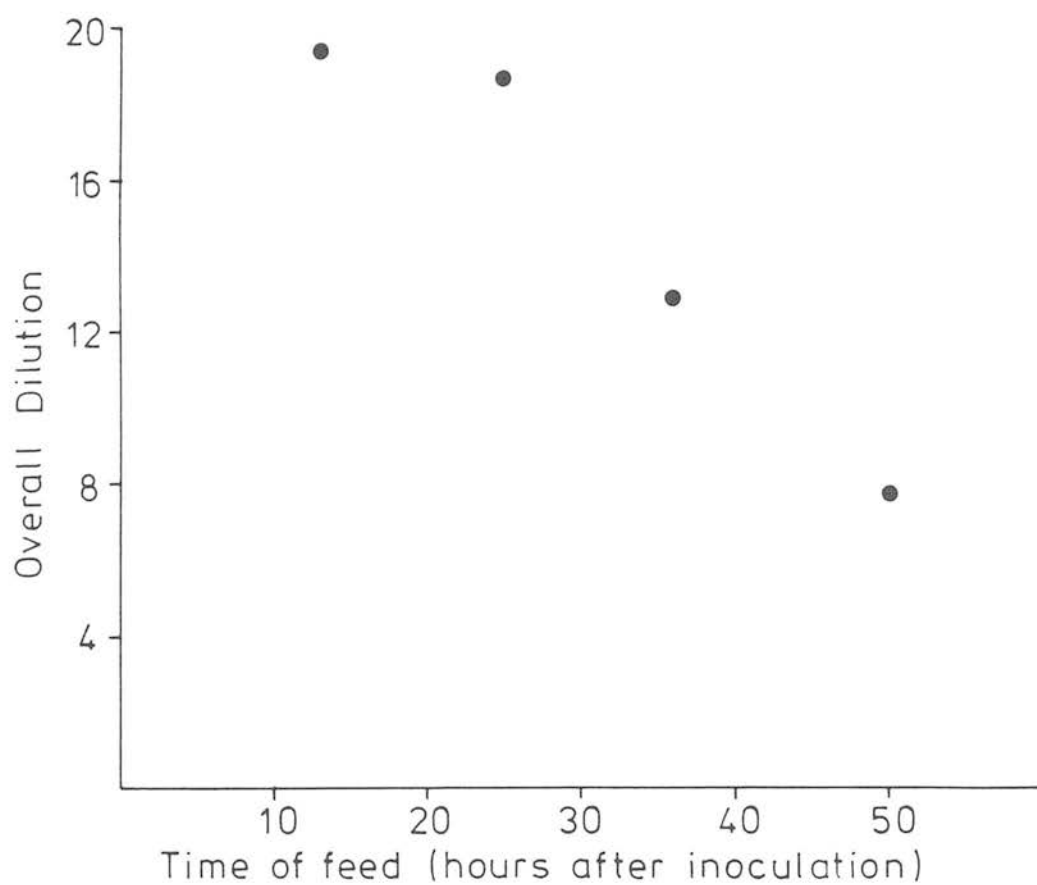
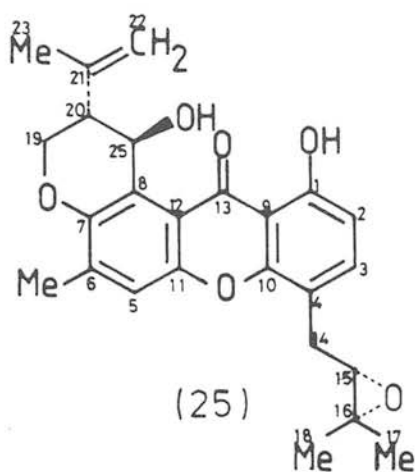


Figure 1 Dilutions of  $[1-^{14}\text{C}]$ acetate in andibenin B



#### 4.2.2 Preliminary Investigations

Preliminary experiments on a strain of *A. variegator*, grown as a surface culture on a liquid medium, indicated that good yields of andibenin B (ca. 100 mg l<sup>-1</sup>) were obtainable. A growth-production study showed that the yield reached a maximum around the 7th day after inoculation. Andilesin A was produced in smaller and variable amounts of about 20 mg l<sup>-1</sup>. Although this latter yield would be acceptable for many purposes, the intention to assess the position of <sup>18</sup>O by <sup>13</sup>C n.m.r. spectroscopy (Section 2.4.3) of labelled samples meant that comparatively large amounts of material were required. Due to the expense of <sup>18</sup>O-labelled precursors, only a limited quantity of fungus could be grown in their presence and so the yield of andilesin A was of some concern.

It was also important to know the expected degree of dilution of labelled acetate by endogenous acetate before carrying out the experiment with <sup>18</sup>O-labelled material. A series of experiments were performed, in which [1-<sup>14</sup>C]-acetate was administered to separate cultures of *A. variegator* at different times after inoculation and the resulting andibenin samples were purified to constant activity. The results are depicted in Figure 1. Of the four times chosen for the feeds, that of 50 hours gave the lowest dilution of label. Unfortunately, this does not necessarily show the best time for feeding as no feeds were given after this time. However, the indicated dilution of 7.7-fold overall or 77-fold per labelled site<sup>5</sup> is satisfactory, providing that

exchange of the  $^{18}\text{O}$  label of the  $[1-^{13}\text{C}, ^{18}\text{O}_2]\text{acetate}$  and of any labelled intermediates is limited. This is something of an unknown factor. Previous studies<sup>19,20,21</sup> have indicated that the degree of exchange is variable but around 50% over the course of biosynthesis for positions other than those which ultimately carry carbonyl groups. These are relatively susceptible to exchange *via* the hydrated form.

The preliminary radiolabelling experiments also indicated toxic effects due to the added acetate. Compared with a control experiment, yields of andibenin B were significantly reduced in all cases. An experiment in which the same overall amount of radiolabelled acetate was given, but as four separate doses over the course of 2 days, was performed concurrently but this gave no advantage of either higher yield or lower dilution.

#### 4.2.3 Incorporation of $^{18}\text{O}$ -Labelled Precursors

Taking, therefore, the latest of the single-dose experiments as a guide, *A. variegata* cultures were fed a sample of  $[1-^{13}\text{C}, ^{18}\text{O}_2]\text{acetate}$  at 50 hours; andibenin B and andilesin A were isolated as usual. Although on this occasion the yields compared well with those of the control culture, evidence of toxicity of the acetate again appeared. At the end of the growth period the mycelium of the experimental culture appeared curled and dying.

A complementary experiment, in which *A. variegator* was grown under an atmosphere of 30%  $^{18}\text{O}_2$ , 70%  $^{14}\text{N}_2$ , was also carried out. The "closed-volume" apparatus (Appendix) was used. Although the fungus appeared to grow normally, and andibenin B and andilesin A were subsequently isolated in typical quantities, an attempt to isolate the small quantity of tajixanthone (25) usually produced by this strain and, indeed, present in the mycelium of the control culture, proved fruitless. Possibly the absence of  $\text{CO}_2$  in the atmosphere affected production; however, later experiments with a different strain of *A. variegator* gave normal xanthone production under similar conditions (see Chapter 5). Both of the metabolites obtained from the fermentation under  $^{18}\text{O}_2$  gave their major parent-ion peak at  $m/z = 434$ , indicating the incorporation of up to and predominantly 4 atoms of  $^{18}\text{O}$  into andibenin B (unlabelled:  $m/z = 426$ ) and up to 3 atoms into andilesin A (unlabelled:  $m/z = 428$ ).

The samples of andibenin B and andilesin A derived from both  $^{18}\text{O}$ -labelling experiments were examined by 100.6 MHz  $^{13}\text{C}$  n.m.r. spectroscopy. In the case of the  $^{18}\text{O}_2$ -labelled samples, unlabelled metabolite was added to provide unshifted signals as internal references against the very small isotope shifts.  $\text{CDCl}_3$  was used as the n.m.r. solvent except for the spectrum of  $^{18}\text{O}_2$ -labelled andibenin B; here  $\text{CD}_2\text{Cl}_2$  was used to avoid obscuring the C-10 resonance which, in  $\text{CDCl}_3$ , occurs at 77.4 p.p.m. Sections of the n.m.r.



Table 1     Isotopically shifted resonances observed in the  
100.6 MHz  $^{13}\text{C}$  n.m.r. spectra of andibenin B (1)  
and andilesin A (4)

Carbon	Andibenin B			Andilesin A		
	$\delta_{\text{C}}^{\text{a}}$	$\Delta\delta_{\text{C}}^{\text{b}}$	$^{16}\text{O}:^{18}\text{O}^{\text{c}}$	$\delta_{\text{C}}^{\text{a}}$	$\Delta\delta_{\text{C}}^{\text{b}}$	$^{16}\text{O}:^{18}\text{O}^{\text{c}}$
4, <sup>d</sup>	214.2	5.0	81:19	215.0	5.2	84:16
8, <sup>d</sup>	167.6	3.6	91:9	174.7	3.9	89:11
8, <sup>e</sup>	167.5 <sup>f</sup>	1.2	68:32	174.7	1.2	90:10
3 <sup>e</sup>	163.9 <sup>f</sup>	4.7	80:20	166.3	4.6	85:15
4 <sup>e</sup>	85.4 <sup>f</sup>	4.3	67:33	83.6	4.8	89:11
10 <sup>e</sup>	77.9 <sup>f</sup>	3.1	72:28			
6, <sup>e</sup>				72.1	?	
1, <sup>e</sup>	68.9 <sup>f</sup>	2.9	70:30	69.3	2.9	88:12

<sup>a</sup> Chemical shifts ( $\text{CDCl}_3$ ) in p.p.m. downfield from TMS  
but see below<sup>f</sup>

<sup>b</sup> Isotope shift in p.p.m./100, upfield from unshifted signal

<sup>c</sup> Intensity ratio of unshifted to shifted signal, obtained  
from a Dupont 210 curve analyser ( $\pm 5\%$ )

<sup>d</sup> Shift due to  $[1-^{13}\text{C}, ^{18}\text{O}_2]\text{acetate}$

<sup>e</sup> Shift due to  $^{18}\text{O}_2$  gas

<sup>f</sup> In  $\text{CD}_2\text{Cl}_2$ , referenced to  $\delta_{\text{C}} = 53.6$  p.p.m.

spectra are shown in expanded form in Figure 2 (andibenin B) and Figure 3 (andilesin A) and the results are summarised in Table 1.

The  $^{13}\text{C}$  n.m.r. spectral assignments are those previously published<sup>4,5</sup> in the case of andibenin B. Those of andilesin A which are relevant to these experiments follow easily from single-frequency, off-resonance, proton-decoupling (s.f.o.r.d.) data and from comparisons with the spectra of andibenin B and of the other andilesins<sup>4</sup>: Two signals, due to the lactone carbonyls, which in andibenin B (1) occur at 167.6 p.p.m. (C-8') and 164.4 p.p.m. (C-3,  $\text{CDCl}_3$ ) appear at 174.7 p.p.m. and 166.1 p.p.m. in the spectrum of andilesin A (4). The difference of at least 7.1 p.p.m. downfield shown by the former signal is presumably due to the absence of conjugation of this carbonyl, *i.e.* C-8'. The downfield position cannot be explained by the difference in the size of the ring-A lactone as andilesin B<sup>4</sup> (5) shows two signals in this spectral region at 166.2 p.p.m. and 166.6 p.p.m.

Andilesin A (4) shows three signals: at 83.6, 72.1 and 69.3 p.p.m., due to carbons bearing only singly-bonded oxygen, which appear as a singlet, a doublet and a triplet respectively under s.f.o.r.d. conditions, assigning them to C-4, C-6' and C-1' in that order. Those signals due to C-4 and C-1' also appear in very similar positions to the corresponding resonances in the spectra of andibenin B (1) and of andilesins B (5) and C (6)<sup>4</sup> whereas that due to C-6', as expected, does not.

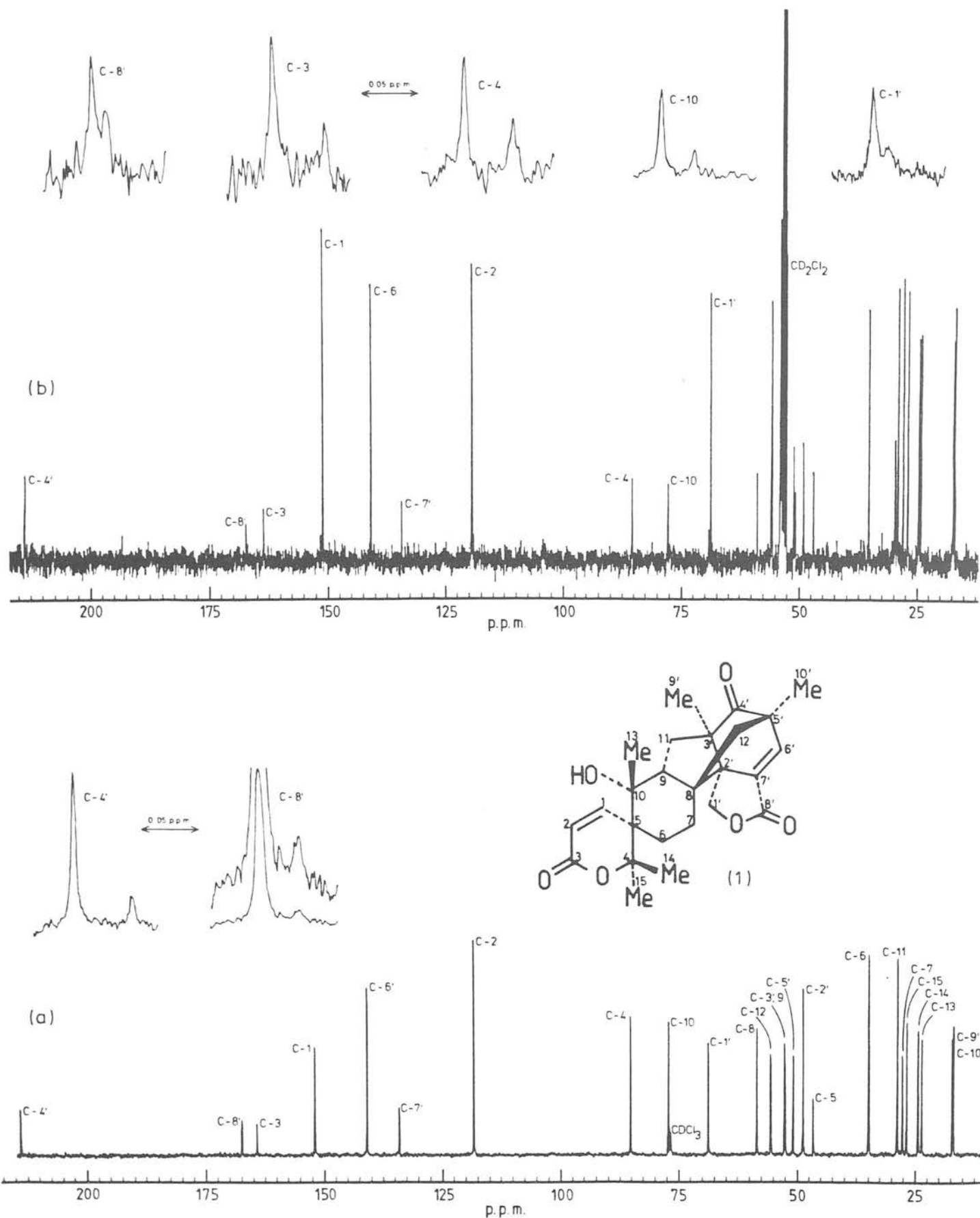


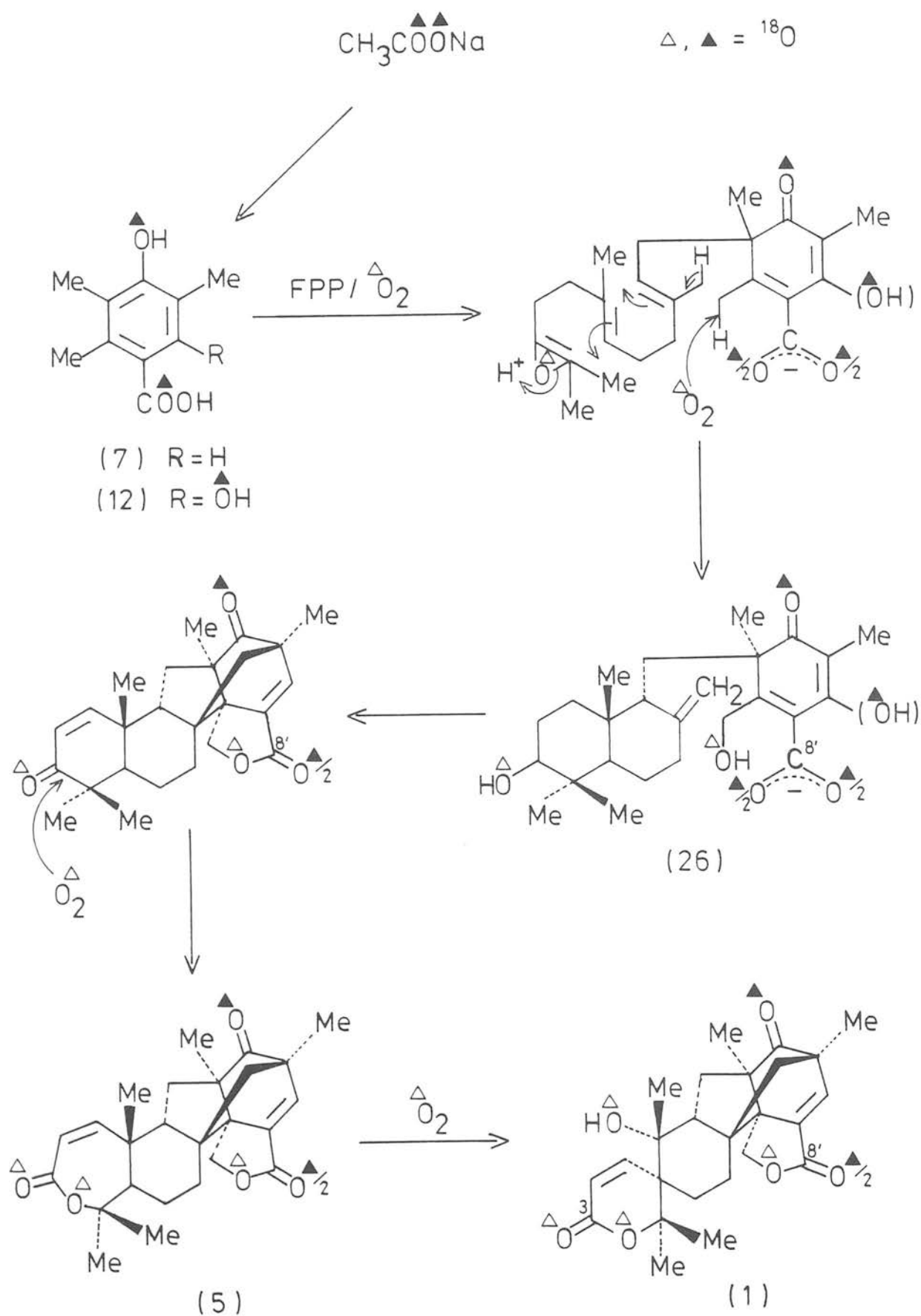
Figure 2

Sections of the 100.6 MHz  $^{13}\text{C}$  n.m.r. spectra of andibenin B: (a) labelled by  $[1-^{13}\text{C}, ^{18}\text{O}_2]$ acetate; (b) labelled by  $^{18}\text{O}_2$  gas

#### 4.2.4 Results: Andibenin B

The andibenin B spectra showed shifted signals due to atmospherically-derived  $^{18}\text{O}$  for carbons 8', 3, 4, 10 and 1'. As the isotope shift of the C-3 resonance is far too large<sup>22,23</sup> to be caused solely by  $^{18}\text{O}$ -labelling of the singly-bonded lactone oxygen, the C-3 carbonyl oxygen must carry  $^{18}\text{O}$ . Although the C-3 isotope shift could be wholly accounted for by this label, the existence also of the C-4 shift shows that both of the  $\delta$ -lactone oxygens are derived from  $\text{O}_2$  gas. C-8' shows a shift of 0.012 p.p.m. which is too small<sup>22,23</sup> to be due to the carbonyl oxygen and, with the observation of a shift for C-1', indicates the atmospheric origin of the singly-bonded  $\gamma$ -lactone oxygen. The C-10 hydroxyl oxygen is also derived from the atmosphere.

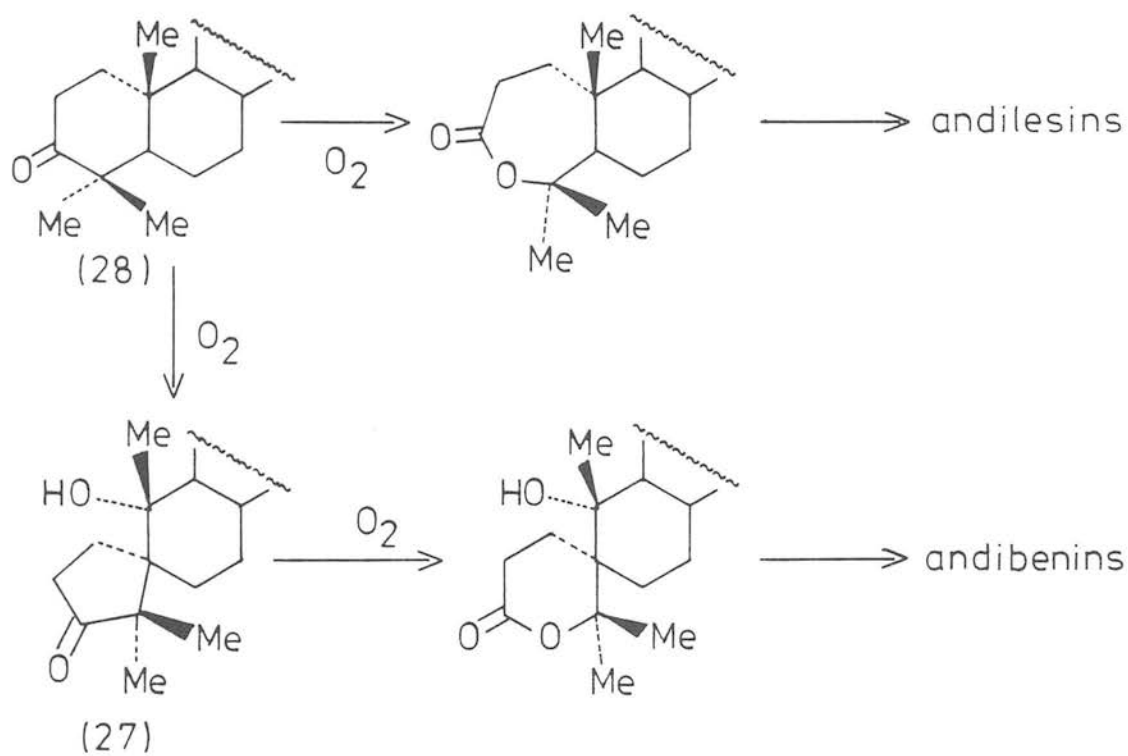
Andibenin B produced in the presence of  $^{18}\text{O}$ -labelled acetate showed shifted signals for C-4' and C-8' only. This time the C-8' shift was much larger (0.036 p.p.m.) and therefore due to labelling of the doubly-bonded oxygen. For both these acetate-derived oxygens the retention of  $^{18}\text{O}$  label seems fairly low. Although the spectra were obtained under conditions<sup>29,30</sup> conducive to high resolution rather than accurate intensities and the observed line heights are therefore unreliable, visual comparison of the unexpanded spectra (Figure 2) obtained after the two fermentations (with  $^{18}\text{O}_2$  gas and with  $^{18}\text{O}$ -labelled acetate) indicates that  $^{13}\text{C}$  incorporation into the positions<sup>5</sup> derived from C-1



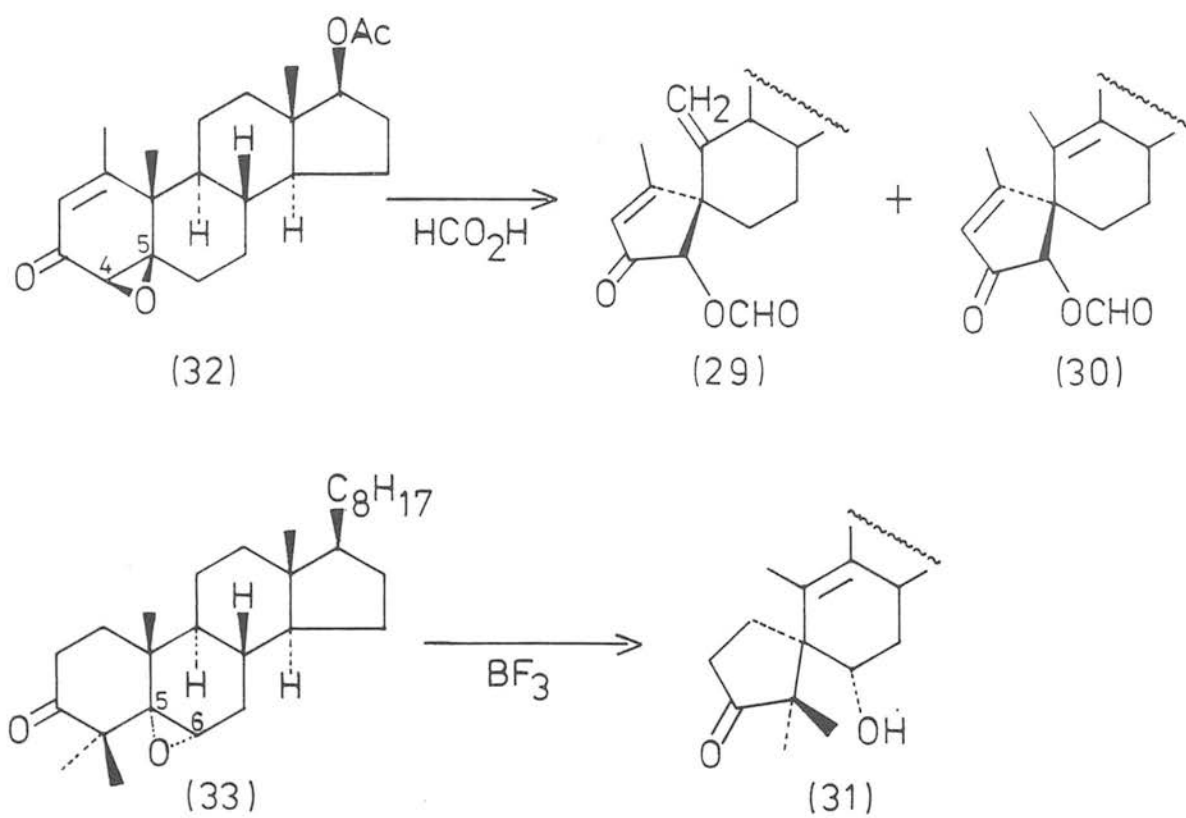
Scheme 5      Oxygen - 18    labelling of andibenin B

of acetate is of the order of 1%, as predicted by the  $^{14}\text{C}$  experiments. As the acetate was labelled with  $^{13}\text{C}$  and  $^{18}\text{O}$  to the extent of 90% in each case, the results indicate that about 40% of the  $^{18}\text{O}$  at C-4' and about 20% of that at C-8' is retained. These two figures are only very rough estimates of absolute retention but their relative sizes are consistent with the intermediacy of an orsellinate (or 2-deoxyorsellinate) species in which the future C-8' forms a free carboxylic acid, and later loses half of its  $^{18}\text{O}$  label on lactonisation. Exchange processes presumably account for the rest of the lost  $^{18}\text{O}$ .

The results of these experiments indicate a pathway such as shown in Scheme 5. An atmospheric origin for the C-3 carbonyl oxygen is unsurprising and suggests that cyclisation of the farnesyl group is promoted by formation of an epoxide such as is usually implicated in the cyclisations of squalene<sup>24</sup>. Formation of the  $\gamma$ -lactone, the ether oxygen of which is atmospherically derived, is likely to require a prior oxidation of the tetraketide "starter" methyl. In Scheme 5 a hydroxymethyl species (26) is postulated, in which the sesquiterpene residue is present, but there is no evidence for the stage at which this oxidation and subsequent lactonisation occur: the  $\gamma$ -lactone might form before alkylation with farnesyl pyrophosphate. However, the labelling experiments do indicate that in this process it is the C-8' carboxyl which undergoes nucleophilic attack rather than the hydroxymethyl group.



Scheme 6

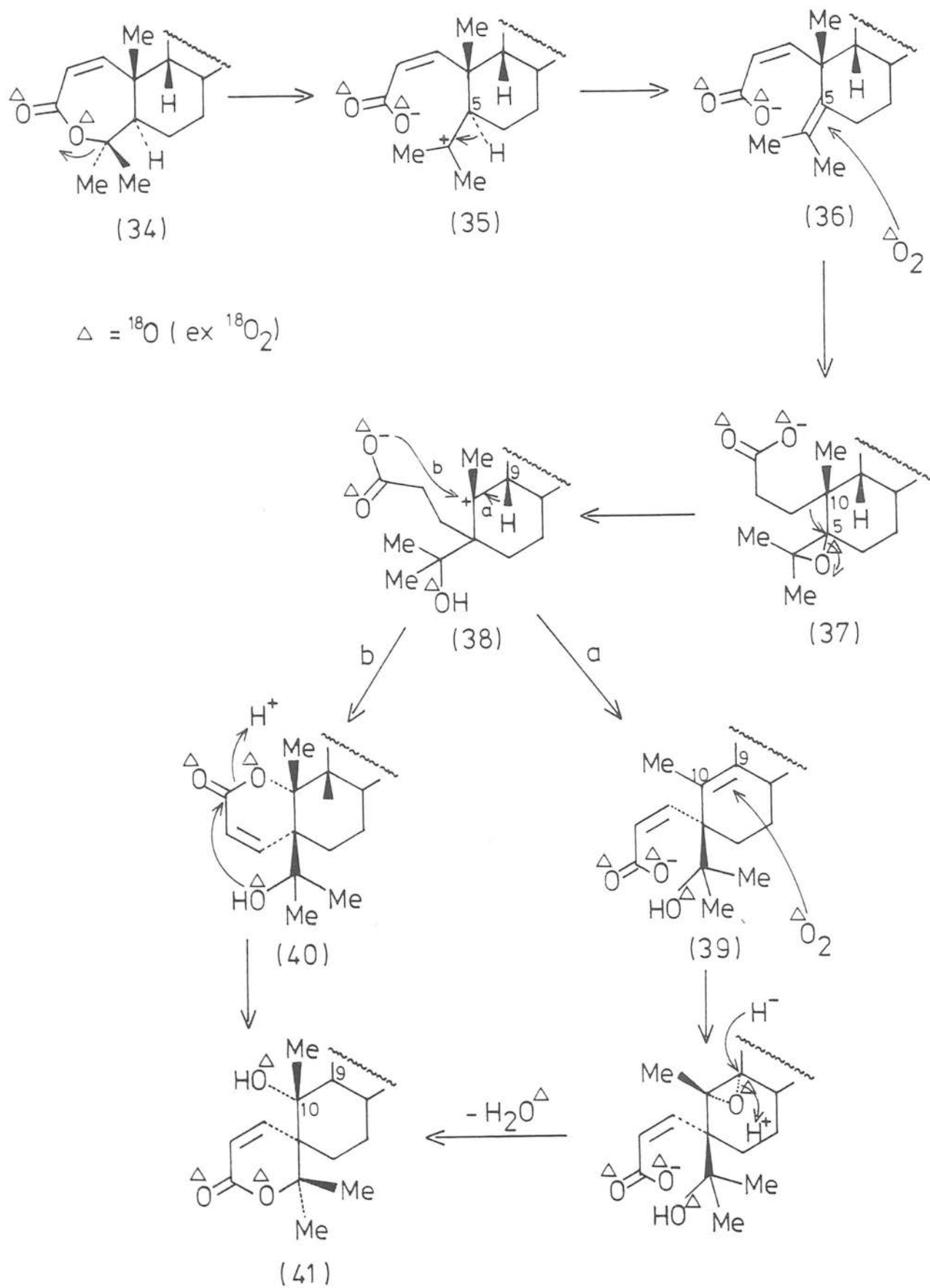


Scheme 7

The presence of oxygen of atmospheric origin attached to C-4 and C-10 throws some light on possible mechanisms for the biosynthesis of the spiro-lactone system. As the andilesins, plausible intermediates of the andibenins, possess the 7-membered lactone ring, it seems reasonable to suppose that the required skeletal rearrangement to form the spiro-lactone occurs subsequent to the introduction of this C-4 oxygen. This could alternatively be a manifestation of a non-specific mono-oxygenase system mediating a Baeyer-Villiger type oxidation<sup>25</sup> on both spirocyclo-pentanone (27) and cyclohexanone (28) species (Scheme 6). However, some form of activation is required before a rearrangement of this nature can occur. Similar rearrangements of steroids are observed when a good leaving group is present at C-5 (Scheme 7). Spiro compounds (29), (30), (31) were formed on the treatment of the 4 $\beta$ , 5 $\beta$  epoxide (32) with formic acid<sup>26</sup>, or of the 5 $\alpha$ , 6 $\alpha$  epoxide (33) with boron trifluoride<sup>27</sup>.

On paper at least, it is possible to derive such a leaving group from the andilesin lactone ring (34) (Scheme 8). A relatively stable carbocation (35) could be generated from the ester link by alkyl-oxygen cleavage which, under acid conditions at least, is the predominant cleavage mechanism for esters of tertiary alcohols<sup>28</sup>. If quenching by water is avoided, loss of the C-5 proton would then give the tetrasubstituted alkene (36) by a net elimination.





Scheme 8

Insertion of atmospheric oxygen by a mono-oxygenase could then provide the epoxide (37) in which the required bond migration to C-5 would be promoted. The carbocation (38) thus generated at C-10 cannot be quenched by water as the eventual 10-hydroxyl group of andibenin B is labelled by  $^{18}\text{O}_2$ . An elimination to the 9,10 alkene (39) by loss of the C-9 proton and subsequent mono-oxygenase epoxidation and hydride reduction (route a) would explain the observations. This route has the advantage that *any* subsequent esterification mechanism for formation of the  $\delta$ -lactone ring will result in two oxygens of atmospheric origin in the ester link. The structure of austin (17) which contains a 9,10 double-bond also provides circumstantial support for this pathway.

Route b provides an alternative mechanism whereby the C-10 carbocation (38) is trapped by the carboxyl group to form a lactone (40) which then undergoes transesterification giving the A and B rings (41) of andibenin B directly. In this case it is not necessary to invoke a further atom of atmospheric oxygen to account for the labelling of the 10-hydroxyl. The transesterification, required if the introduction of water-derived oxygen into the ester-link is to be avoided, would have to involve acyl-oxygen bond cleavage. As the 10-hydroxyl is tertiary, this might imply a base-catalysed mechanism.

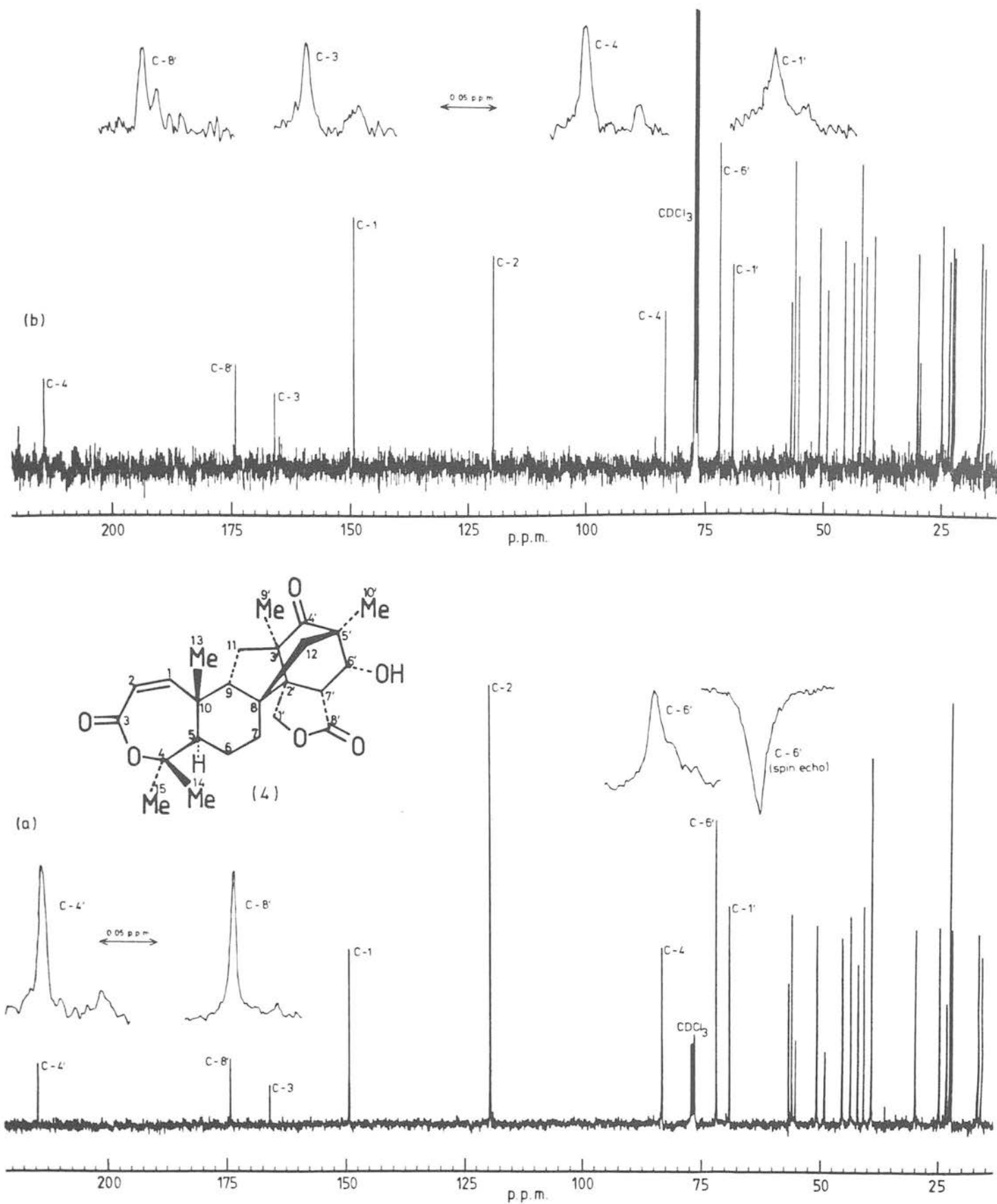
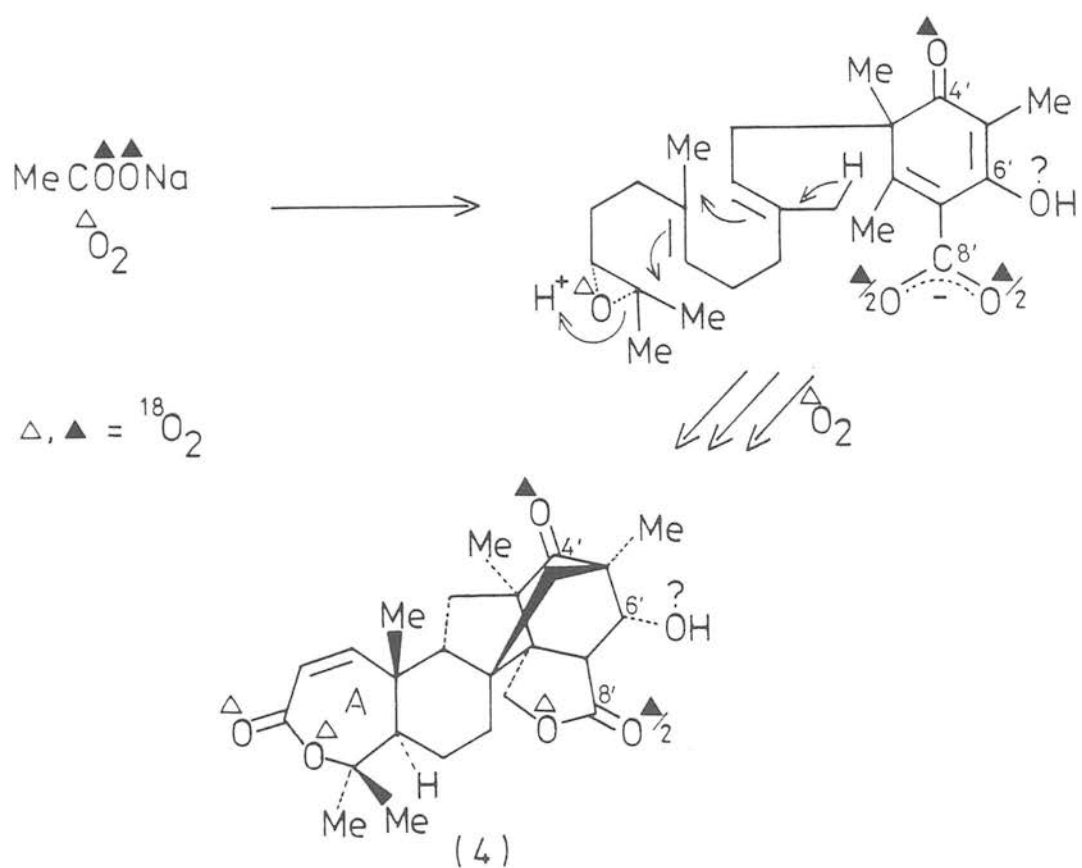


Figure 3 Sections of the 100.6 MHz  $^{13}\text{C}$  n.m.r. spectra of andilesin A: (a) labelled by [1- $^{13}\text{C}$ ,  $^{18}\text{O}_2$ ]acetate; (b) labelled by  $^{18}\text{O}_2$  gas



Scheme 9 Oxygen - 18 labelling of andilesin A

These two routes, in principle, could be distinguished by an experiment to check the origin of the C-9 proton.

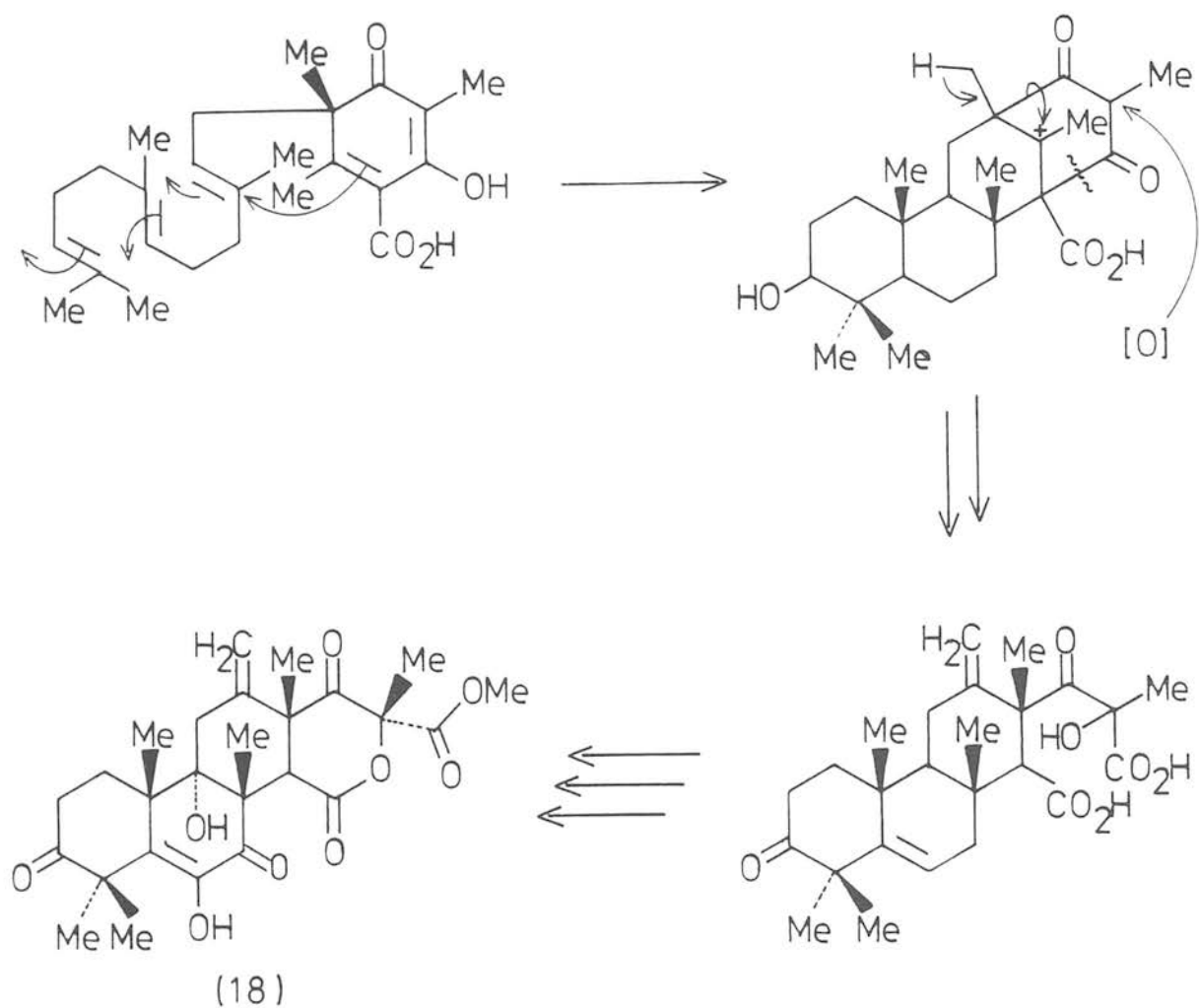
[4-<sup>2</sup>H<sub>2</sub>]Mevalonic acid would label andibenin B at this position only if a mechanism such as route b was operative.

#### 4.2.5 Results: Andilesin A

The oxygen-containing functionalities of andilesin A (4) which are common to it and andibenin B show the same distribution of labelling from the <sup>18</sup>O experiments (Scheme 9). Thus the C-4' and C-8' carbonyl oxygens are both acetate-derived and the latter again shows the lesser retention of label. Oxygen gas labelled both the ring-A lactone oxygens and the singly-bonded oxygen of the γ-lactone. Disappointingly, the results obtained so far for the oxygen atom of most interest, *i.e.* that of the 6'-hydroxyl group, are not so clear cut. Expansion of the normal <sup>13</sup>C spectrum of andilesin A enriched by [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate showed an up-field shoulder on the C-6' signal (Figure 3). However, the application of a pulse sequence<sup>29,30</sup> designed to eliminate any long range carbon-carbon coupling arising from the adjacent incorporation of labelled acetate units resulted in a single clean signal. This might be taken as evidence against an acetate origin for the 6'-hydroxyl but, at the estimated incorporation level of about 1% (see above), long range coupling should have been negligible anyway and another explanation for the original shoulder may be required. Due to the small quantity of material available

and the low retention of acetate-derived oxygen, the spectra of this sample are of poor quality generally and do not encourage firm conclusions.

Nevertheless it is clear that the 6'-hydroxyl is not labelled from  $^{18}\text{O}_2$  gas. In this case C-6' shows no trace of a shifted signal and, even should such a shift be too small to resolve, the fact that the three atoms of  $^{18}\text{O}$  detected by mass-spectrometry are all otherwise accounted for would allow this conclusion anyway. In addition, an M-18 peak in the mass-spectrum of unlabelled andilesin A, presumably due to loss of the 6'-hydroxyl as water, is also present in the mass-spectrum of andilesin A labelled from  $^{18}\text{O}_2$ , albeit at  $m/z = 416$ , 6 mass-units heavier.



Scheme 10 Hypothetical triprenyl-phenol origin of territonin

### 4.3 Biosynthetic Studies on Terretonin

#### 4.3.1 Preamble: Object of Experiments

The biosynthetic work on anditomin<sup>10</sup> (16) and austin<sup>14,15</sup> (17) has shown that the triprenyl-phenol pathway need not be restricted to metabolites which retain intact the carbon ring derived from the phenol residue. In the case of anditomin, the <sup>13</sup>C-labelling pattern could be explained by a rearrangement (Scheme 3, Section 4.1) whereby the bond between C-3' and C-4' was broken. A <sup>1</sup>J<sub>CC</sub> coupling between these two carbons, in the <sup>13</sup>C n.m.r. spectrum of anditomin labelled by [1,2-<sup>13</sup>C<sub>2</sub>]acetate, was absent. The same coupling was lost in the corresponding experiment with austin although in this case the bond was broken by oxygen insertion. In addition, the structure of austin requires a rearrangement whereby the bond originally between C-4' and C-5' is replaced by one between C-4' and C-6' (Scheme 4, Section 4.1).

When terretonin (18), C<sub>26</sub>H<sub>32</sub>O<sub>9</sub>, was first isolated from *Aspergillus terreus*<sup>13</sup>, its formation by oxidative modification of a triterpene intermediate was suggested. Elements of its structure, however, were reminiscent of the *A. variegator* triprenyl-phenol metabolites, and a biogenesis along similar lines was proposed<sup>10</sup>. Again, cleavage of the tetraketide-derived ring would be necessary to account for the structure (Scheme 10). The object of the experiments described below was to test this latter proposal, initially by the incorporation of simple, <sup>13</sup>C-labelled precursors.



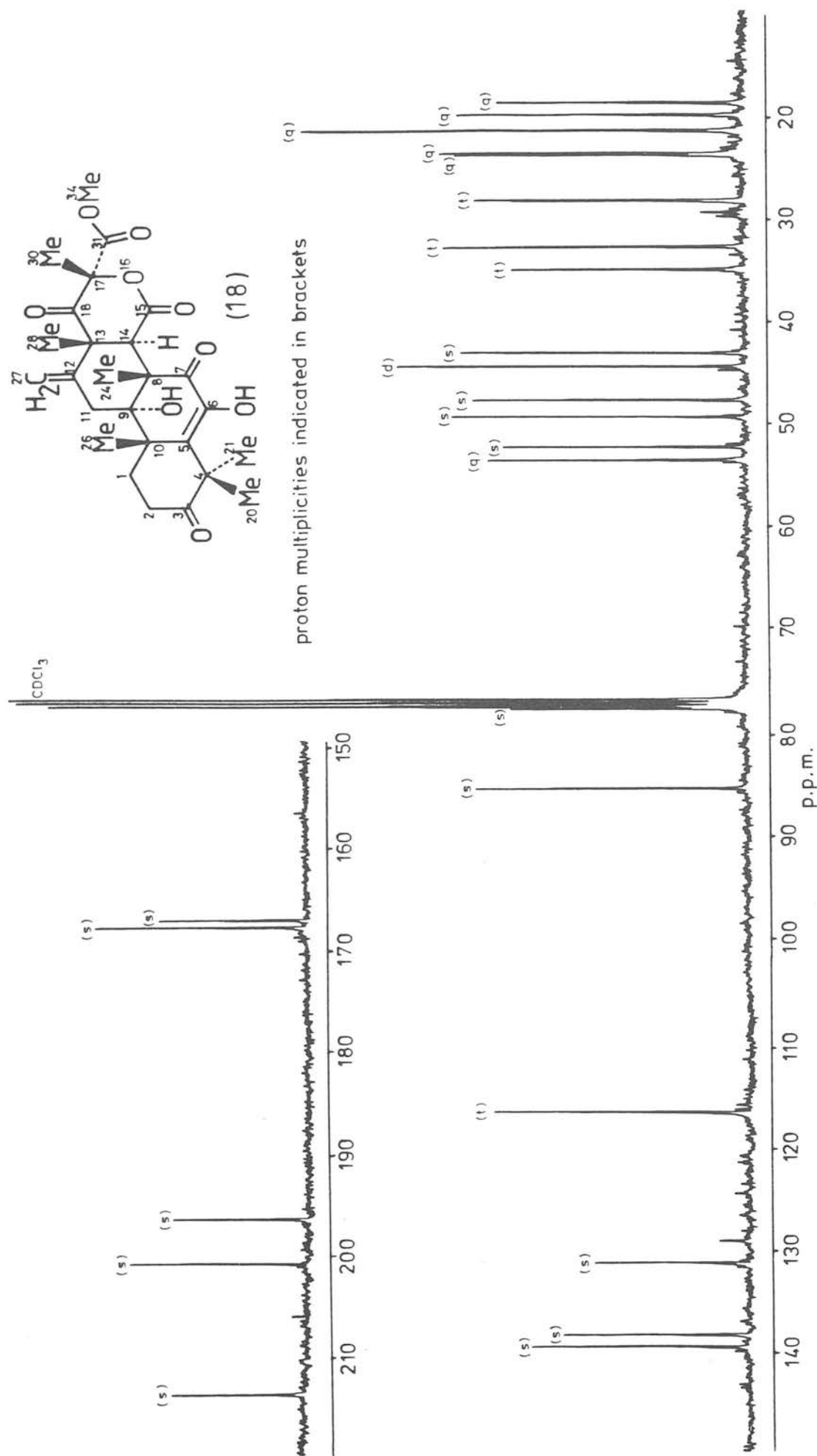


Figure 4 The 90.6 MHz  $^{13}\text{C}$  n.m.r. spectrum of terretonin (in  $\text{CDCl}_3$ )

#### 4.3.2 Production of Terretonin

Early experiments, to establish suitable culture conditions for the production of terretonin by *A. terreus*, were carried out using the medium as previously published<sup>13</sup>. The fungus grew well and a small quantity of terretonin was eventually isolated. However, the work-up was very protracted, owing largely to the presence in the medium of "Shredded Wheat"! As this medium was virtually solid, it was also unsuitable for any feeding studies where the labelled substrate was to be added after inoculation. Later experiments used a liquid medium, based on malt-extract broth, from which the isolation procedure proved very easy. Reliable yields of 30-35 mg l<sup>-1</sup> of terretonin were obtained from this medium.

#### 4.3.3 Assignment of the <sup>13</sup>C N.M.R. Spectrum

A rigorous assignment of the <sup>13</sup>C n.m.r. spectrum of terretonin (Figure 4) was initially required. Although a partial assignment, to show consistency with the crystal structure, had been made<sup>13</sup>, this was based only on chemical shift data and single-frequency, off-resonance, proton-decoupled (s.f.o.r.d.) spectra and was not reliable enough for <sup>13</sup>C-labelling studies. Chemical shifts and proton multiplicities (Figure 4) alone, do, however, allow the confident assignment of three of the <sup>13</sup>C signals of terretonin: that of the methine carbon (C-14), that of the exocyclic methylene carbon (C-27) and that of the

methoxyl carbon (C-34). The signal at 44.4 p.p.m. (C-14) is the only one appearing as a doublet under s.f.o.r.d. conditions, and the chemical shift (116.7 p.p.m.) sets the C-27 signal apart from those of the other methylene groups. Likewise, C-34 is assigned to the quartet signal at 53.5 p.p.m.

The other 23 resonances can be divided into various groups, based on their chemical shifts<sup>31</sup> and proton multiplicities. Their appearance as quartets in the s.f.o.r.d. spectrum confirms that the six signals at highest field belong to the six C-methyls. Three triplet signals, at 27.9, 32.5 and 34.7 p.p.m., are allocated to the  $sp^3$  methylene groups. All of the remaining signals are singlets under s.f.o.r.d. conditions and those four below 53 p.p.m. can be assigned, unequivocally as a group, to the four unfunctionalised quaternary positions: C-4, 8, 10 and 13. Two signals, at 77.5 and 85.3 p.p.m., each belong to one or other of the non-protonated, oxygen-bearing,  $sp^3$  carbons, *i.e.* C-9 and C-17. Three non-protonated alkene positions: C-5, 6 and 12, are present and the signals at 131.5, 138.5 and 139.6 p.p.m. are assigned thereto. The remaining five carbonyl signals can be divided, on chemical shift grounds, into those of the two ester carbonyls, C-15 and C-31, at 167.6 and 168.3 p.p.m., and those three at lowest field, due to the three ketone carbonyls.

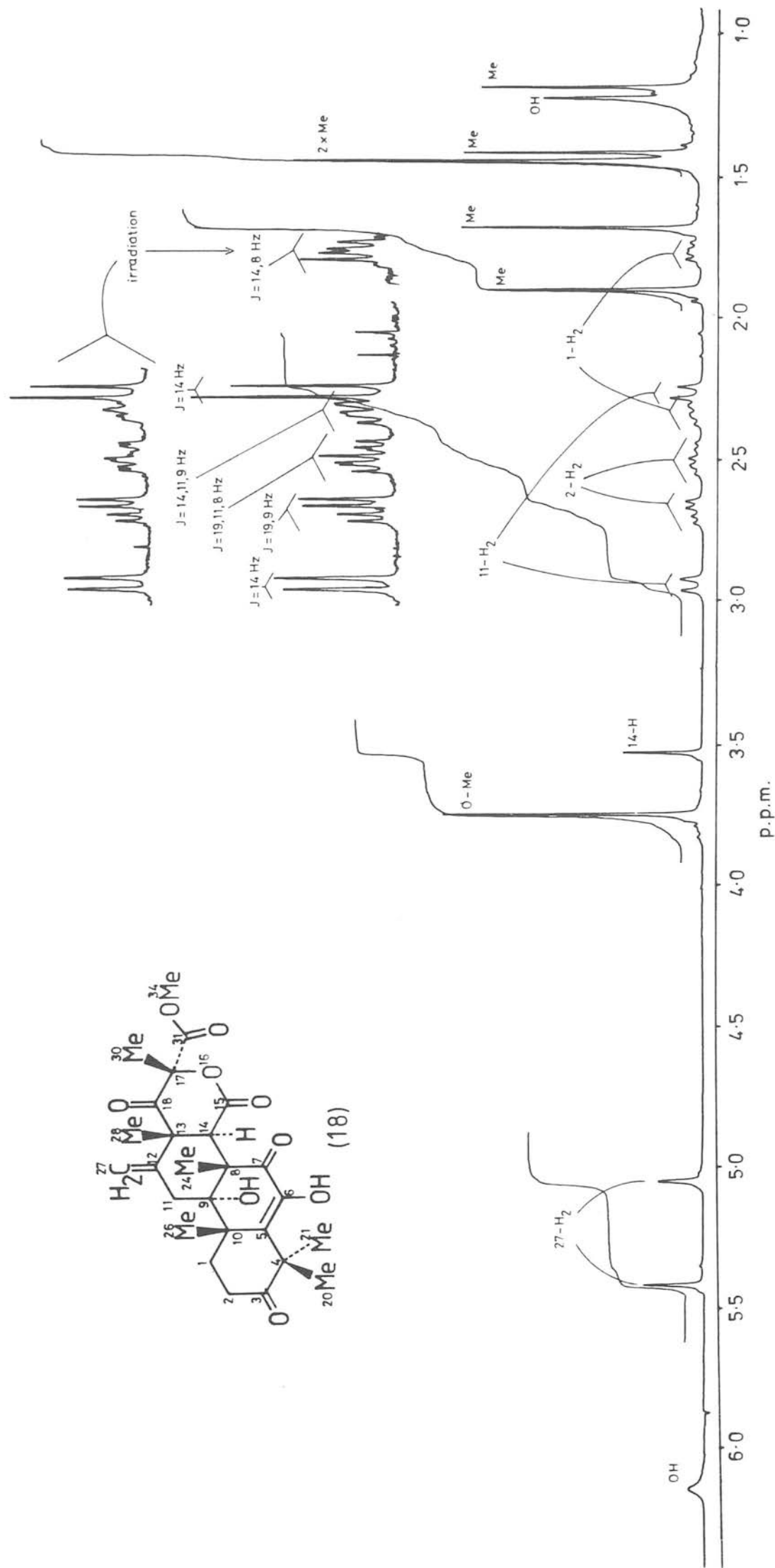
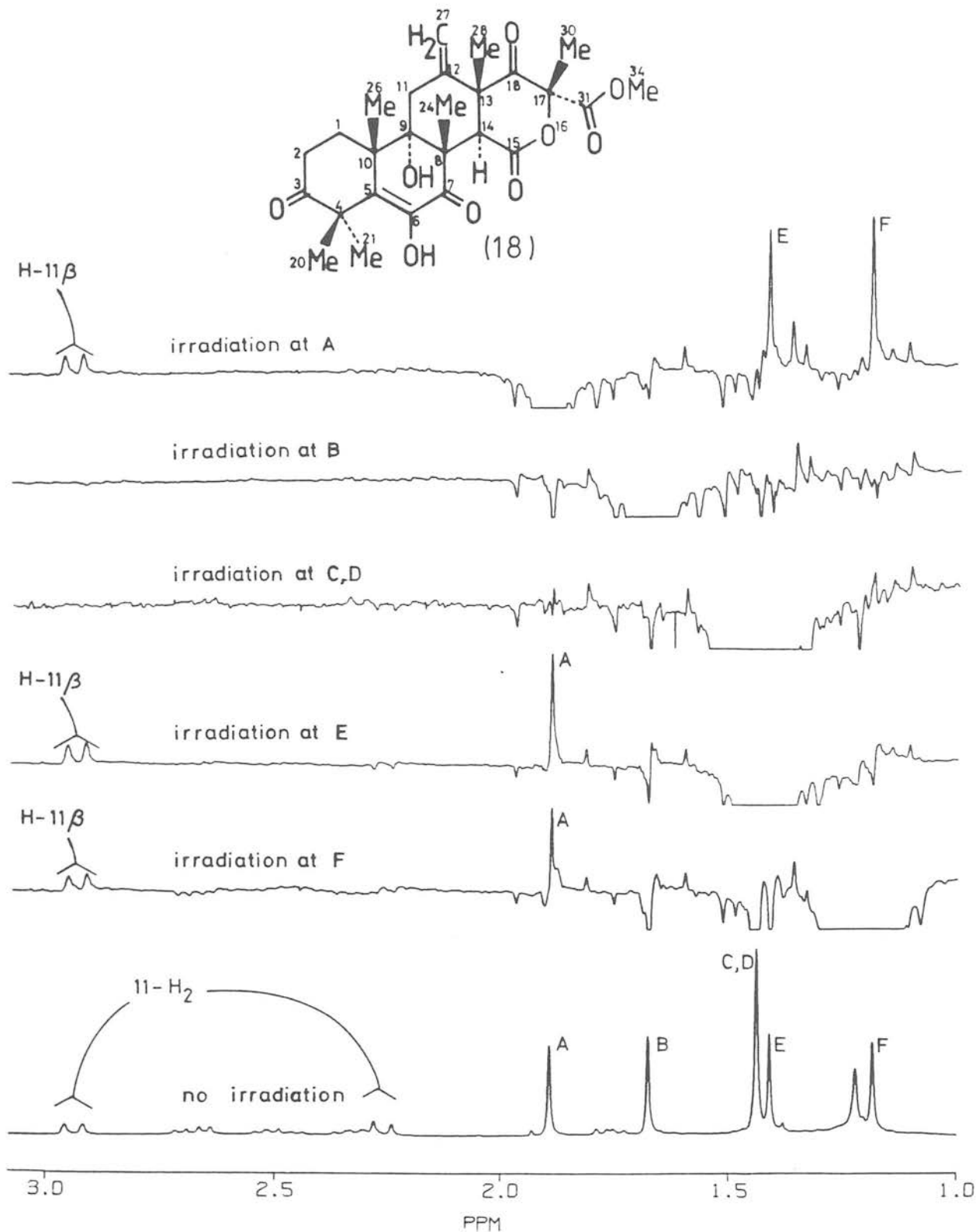


Figure 5 The 360 MHz  $^1\text{H}$  n.m.r. spectrum of terretonin (in  $\text{CDCl}_3$ )

The  $^{13}\text{C}$  signals of the two hydroxylated carbon atoms, C-6 and C-9, were identified from the comparison of p.n.d.  $^{13}\text{C}$  n.m.r. spectra obtained before and after adding a little of a 50:50 mixture of  $\text{D}_2\text{O}$  and  $\text{H}_2\text{O}$ . Providing proton-deuteron exchange occurs but is sufficiently slow on the n.m.r. timescale, this device results in an observable, isotopically-induced splitting in the  $^{13}\text{C}$  signals of carbon atoms bearing hydroxyl groups<sup>32,33</sup>. To avoid obscuring the terretonin signal at 77.5 p.p.m. with the solvent peaks of  $\text{CDCl}_3$ , these spectra were obtained using  $\text{CD}_2\text{Cl}_2\text{-d}_6$  DMSO, 70:30 as solvent: this had a negligible effect on the positions of the spectral lines of terretonin. The addition of the  $\text{D}_2\text{O-H}_2\text{O}$  mixture affected only the signals at 77.5 p.p.m. (77.5 p.p.m. in  $\text{CDCl}_3$ ) and at 139.0 p.p.m. (138.5 p.p.m.); in each case the relative line heights were roughly halved. At the available resolution, the anticipated splittings could not be seen. Taken in conjunction with the chemical shifts, this observation allows the assignment of these two signals to C-9 and C-6 respectively. By default therefore, the signal at 83.9 p.p.m. must be assigned to the remaining non-protonated, oxygen-bearing,  $\text{sp}^3$  carbon, *i.e.* C-17.

Further  $^{13}\text{C}$  assignments were made by recourse to double-resonance experiments involving the proton n.m.r. spectrum. This is shown in Figure 5. The assignment of the proton spectrum is straightforward<sup>13</sup>, to the extent that the signals due to the methine proton, the methoxyl group and the four methylene groups are all readily identifiable



**Figure 6** Difference n.O.e. spectra of terretonin resulting from irradiations at the frequencies of the methyl proton resonances

on the basis of their chemical shifts and coupling constants. Which of the protons of each methylene group is at higher field is not obvious but this is not important in assigning the  $^{13}\text{C}$  n.m.r. spectrum. However, the six methyl signals presented a considerable problem in the  $^1\text{H}$  n.m.r. spectrum as indeed they did in the  $^{13}\text{C}$  spectrum.

Five of the methyl groups of terretonin are sited on the  $\beta$  face, and the five quaternary centres bearing them: C-4, 10, 8, 13 and 17, are all separated by only two bonds. Examination of Dreiding models suggested that if, in solution, all four of the rings of terretonin adopted a "chair" conformation (quasi-chair in the case of ring B) then these five  $\beta$ -methyls would be in close proximity in a line along the upper face of the molecule. It might then be possible to trace this line, and identify the nearest neighbours of each individual methyl group, by using a series of specific proton-frequency irradiations and looking for any nuclear Overhauser effects<sup>34</sup> (n.O.e.'s) in the proton spectra of the unirradiated methyls. Figure 6 shows a series of difference n.O.e. spectra obtained in this way. The methyl signals have been labelled A to F for reference purposes; the unlabelled signal at 1.22 p.p.m. is probably due to the 9-hydroxyl proton which usually appears in this region of the spectrum. That at 1.44 p.p.m. is assigned to two methyl groups, partly by its relative size, but mainly because it correlates to two carbon signals in a two-dimensional, carbon-hydrogen plot (see below). It is

apparent that the irradiations at B or C,D had no significant effect on the peak heights of any of the other signals. Irradiation at A, however, produced a positive difference in the signals E and F. The lower-field resonance of the 11-methylene protons was also enhanced. When either E or F were irradiated, the effect in each case was to increase the intensities of signal A and the downfield H-11 signal. This latter signal must therefore be due to H-11 $\beta$ . Methyls A, E and F are all shown to be close enough to the 11 $\beta$  hydrogen to give rise to an observable n.O.e. so signals A, E and F must each be assigned to one of the three methyl groups at C-24, C-26 and C-28. As methyl A affects, and is affected by, methyls E and F which, however, do not significantly affect each other, methyl A must occupy the central position, *i.e.* C-24, of the three.

No n.O.e. was observed for the other three methyl groups. While this was disappointing from the point of view of the  $^1\text{H}$  n.m.r. spectral assignment, it is consistent with a structure whereby the A and D rings of terretonin adopt "boat" like conformations, presumably to avoid the steric interactions of the methyl groups carried by C-17 and C-4 with their respective neighbouring methyls. This, in fact, is the conformation of the molecule in the crystalline state<sup>13</sup>, so such a situation in solution is not very surprising.



The remaining assignments of signals in the methyl region of the  $^1\text{H}$  n.m.r. spectrum were determined by specific proton-decoupling of the otherwise fully proton-coupled  $^{13}\text{C}$  n.m.r. spectrum (Figure 9). Irradiating proton signal B had a marked effect on three of the carbon resonances including the signal at 85.4 p.p.m. already assigned to C-17 and originally showing a "long range" quartet coupling. This collapsed to a sharp singlet assigning proton signal B to H-30. Proton signals E and F were assigned by the irradiation of signal E: this markedly sharpened the two carbon resonances centered at 44.4 p.p.m. and already assigned to C-14. Of the two possibilities (determined by the n.O.e. experiments) for signal E, *i.e.* H-26 and H-28, only H-28 is close enough to C-14 for its decoupling to have this effect. H-26 is therefore responsible for signal F, the irradiation of which, indeed, had no effect on the C-14 resonances. The double signal, *i.e.* C,D, which is otherwise unassigned, must be due to the gem-dimethyl group.

To summarise, the information so far presented allows the allocation of signals A to F in the proton n.m.r. spectrum as follows:

A	1.89 p.p.m.	H-24
B	1.68 p.p.m.	H-30
C,D	1.44 p.p.m.	H-20, H-21
E	1.41 p.p.m.	H-28
F	1.19 p.p.m.	H-26

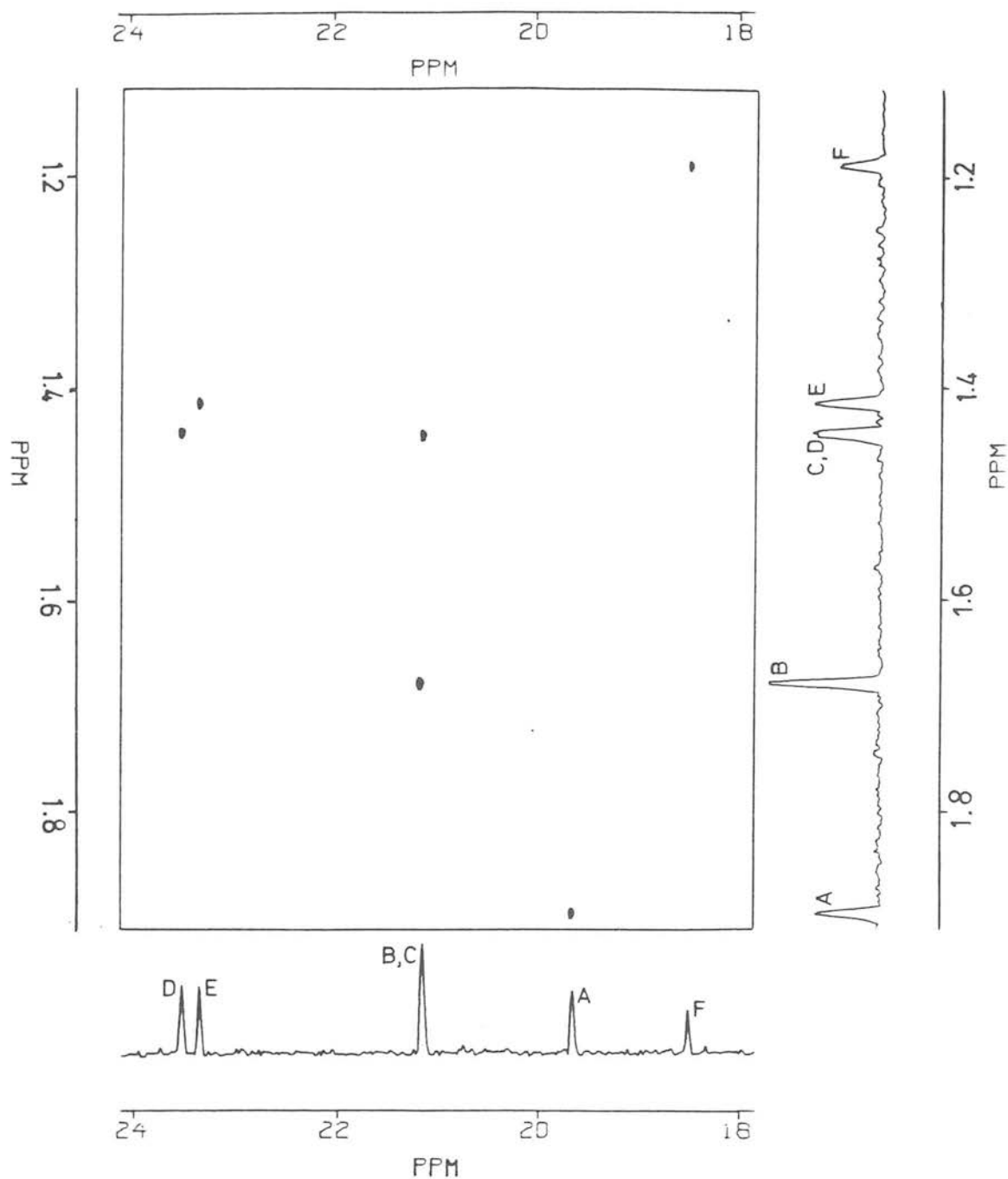


Figure 7 Two-dimensional heteroscalar correlation n.m.r. spectrum (360 MHz and 90.6 MHz) of terretonin (in  $\text{CDCl}_3$ ) matching the  $^{13}\text{C}$  and  $^1\text{H}$  resonances of each methyl group

With these assignments in hand, a two-dimensional, heteroscalar correlation n.m.r. experiment<sup>35</sup> was undertaken in order to match up the <sup>1</sup>H n.m.r. signals in the methyl region with their corresponding <sup>13</sup>C signals. The spectrum is shown in Figure 7. Interestingly, the H-20 and H-21 proton signal at 1.44 p.p.m. correlates to two separate carbon signals, those at 23.5 p.p.m. and 21.1 p.p.m. This latter signal also correlates to the H-30 proton signal at 1.68 p.p.m., confirming that the two signals coincident in the proton spectrum are not from the same two methyl groups which give rise to coincident lines in the carbon spectrum. From the results of this single, powerful experiment, the methyl carbon n.m.r. assignments can now also be summarised:

F	18.4 p.p.m.	C-26
A	19.6 p.p.m.	C-24
B,C	21.2 p.p.m.	C-30 and one of C-20, C-21
E	23.3 p.p.m.	C-28
D	23.5 p.p.m.	one of C-21, C-20

There appears to be no simple way in which the C-20 and C-21 assignments can be rigourously resolved, but fortunately, the point is not crucial to the interpretations of the biosynthetic experiments.

The three sp<sup>3</sup> methylene carbons were assigned by Birdsall-Feeney plots<sup>36</sup>. Because of problems with solubility, these spectra were obtained from a solution in CDCl<sub>3</sub>-d<sub>6</sub> DMSO, 70:30 but the chemical shifts were not significantly affected by this. A series of proton-decouplings, stepped at

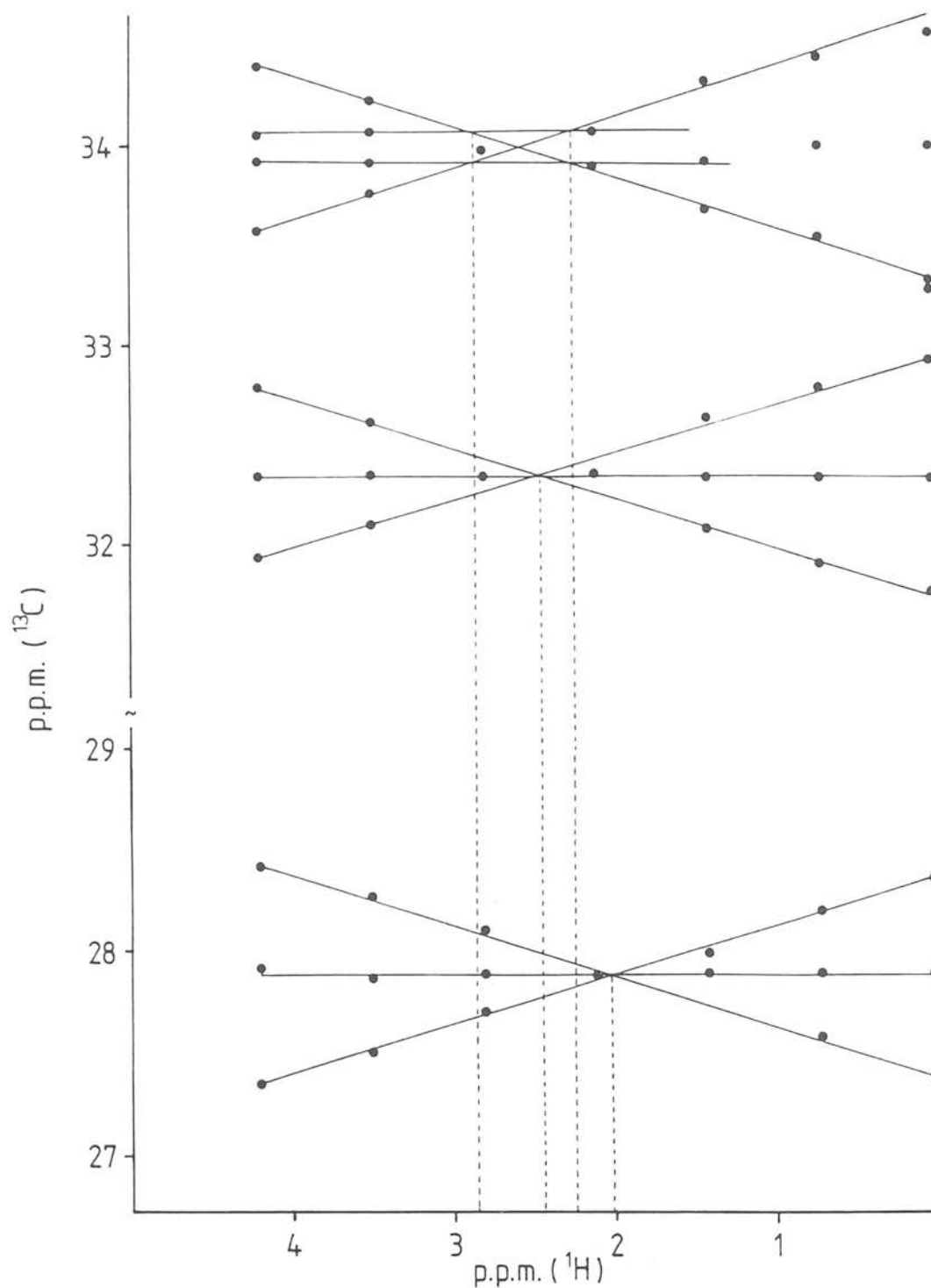


Figure 8

Birdsall-Feeney plots correlating the  $^1\text{H}$  and  $^{13}\text{C}$  n.m.r. resonances of the  $\text{sp}^3$  methylene groups of terretonin (sample in  $\text{CDCl}_3\text{-d}_6\text{-DMSO}$ , 70:30; spectra taken at 360 MHz ( $^1\text{H}$ ) and 90.6 MHz ( $^{13}\text{C}$ ))

Table 2 The effects of specific proton-frequency irradiations on the fully proton-coupled  $^{13}\text{C}$  n.m.r. resonances of the non-protonated carbons of terretonin

$\delta_{\text{C}}$ (p.p.m.) <sup>a</sup>	$\delta_{\text{H}}$ (p.p.m.) <sup>a</sup>						Assignment <sup>b</sup>
	1.19 H-26	1.41 H-28	1.44 H-20/21	1.68 H-30	1.89 H-24	3.53 H-14	
43.0	i						C-10
44.4		sh, f			sh, f?		(C-14)
47.7		sh	sh				C-4
49.3		sh, f	sh, f?			i, sh	C-13
52.2					m-t	i	C-8
77.5							(C-9)
85.3				q-s			(C-17)
131.5							C-5 or 12
138.5							(C-6)
139.6							C-12 or 5
167.6						d-s	C-15
168.3				m-q			C-31
196.8					i, sh	sh, f	C-7
201.2		sh, f		sh, f		sh, f	C-18
213.9							C-3

Key: i = intensity increases  
sh = sharpens  
f = fine structure appears  
d-s = doublet becomes singlet  
q-s = quartet becomes singlet  
m-t = multiplet becomes triplet  
m-q = multiplet becomes quartet

<sup>a</sup> Chemical shifts measured in  $\text{CDCl}_3$  at 360 MHz ( $\delta_{\text{H}}$ ) and 90.6 MHz ( $\delta_{\text{C}}$ )

<sup>b</sup> Assignments in brackets were previously established.

intervals over the range 0-5 p.p.m. were performed and the residual carbon-hydrogen, one-bond couplings were plotted and interpolated to zero (Figure 8). Projection of the zero points onto the proton-frequency axes showed that the carbons with signals at 27.9 p.p.m. (27.9 p.p.m. in  $\text{CDCl}_3$ ) and 32.4 p.p.m. (32.5 p.p.m.) were coupled to the methylene protons with signals centered respectively on 2.02 p.p.m. (2.04 p.p.m.) and 2.46 p.p.m. (2.59 p.p.m.). As the latter proton chemical shift is characteristic of a methylene group adjacent to a carbonyl, the carbon signal at 32.4 p.p.m. is assigned to C-2. Likewise, the signal at 27.9 p.p.m. belongs to C-1. The carbon signal at 34.0 p.p.m. (34.7 p.p.m.) formed a doublet of doublets pattern at some decoupler frequencies and this was correlated to the proton signals at 2.24 p.p.m. (2.26 p.p.m.) and 2.88 p.p.m. (2.94 p.p.m.). Their simple doublet coupling patterns allow the allocation of these two signals to the 11-methylene hydrogens which can only couple significantly to each other; hence the carbon signal at 34.0 p.p.m. is due to C-11.

Further assignments in the  $^{13}\text{C}$  n.m.r. spectrum can be deduced from the specific proton decouplings previously mentioned (Figure 9). Their effects on the fine structure of carbon signals occurring above 40 p.p.m. (with the exceptions of the C-34 methoxyl and C-27 methylene resonances) are summarised in Table 2 and Figure 10 illustrates the long-range carbon-hydrogen interactions thus identified. Apart from the effect on the C-17 resonance (see above), the decoupling of the H-30 protons sharpened the ester carbonyl resonance

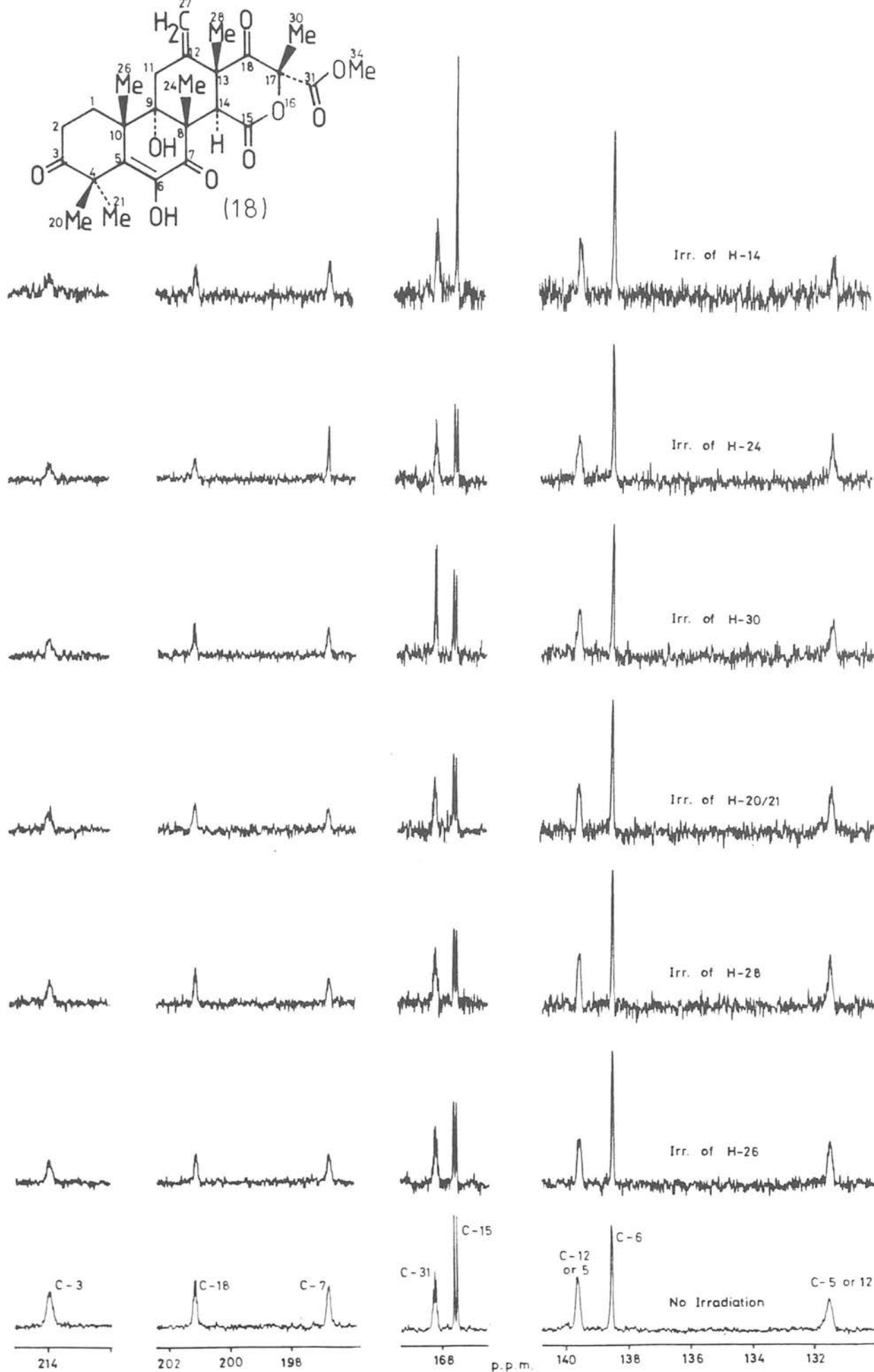


Figure 9

Sections of the fully  $^1\text{H}$ -coupled 90.6 MHz  $^{13}\text{C}$  n.m.r. spectrum of terretonin and the results of selective  $^1\text{H}$ -decoupling experiments (in  $\text{CDCl}_3$ )

at 168.3 p.p.m. from a multiplet to a quartet and introduced fine structure into the ketone carbonyl resonance at 201.2 p.p.m., previously an unresolved envelope of peaks. These two signals must be due respectively, to C-31 and C-18. This is consistent with the results of irradiation at the position of the methine proton resonance (3.53 p.p.m.), upon which the other ester carbonyl signal at 167.5 p.p.m. collapsed from a doublet to a singlet, allocating it to C-15. Confirmation of the C-18 assignment came from decoupling the H-14 methine proton and the H-28 protons: in each case the signal at 201.2 p.p.m. sharpened and developed fine structure.

Only two carbonyl signals remain unaccounted for; those at 196.8 and 213.9 p.p.m. None of the decouplings carried out had a discernable effect on the lower field of these two, but decoupling of H-14 or the H-24 protons in each case sharpened the signal at 196.8 p.p.m. which must therefore be due to C-7. By elimination then, C-3 gives rise to the signal at 213.9 p.p.m.

None of the three endocyclic alkene carbons gave a noticeably modified signal for any of the proton decouplings. That at 138.5 p.p.m. is known to be due to C-6 from the  $D_2O-H_2O$  exchange experiment but those at 131.5 and 139.6 p.p.m., which each belong to one of C-5 or C-12, are not distinguished further.



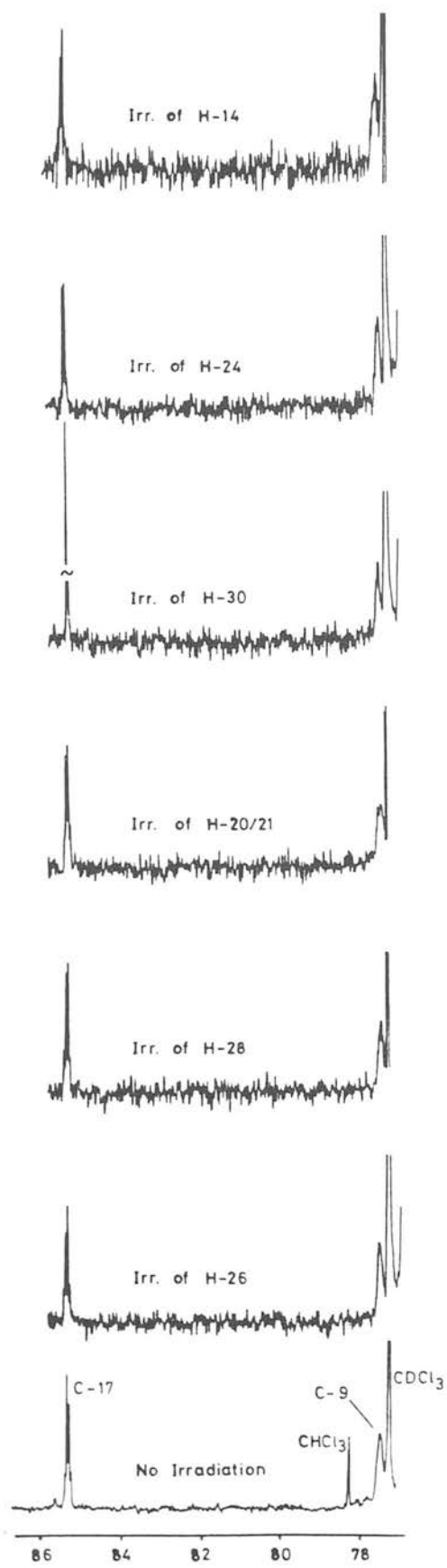
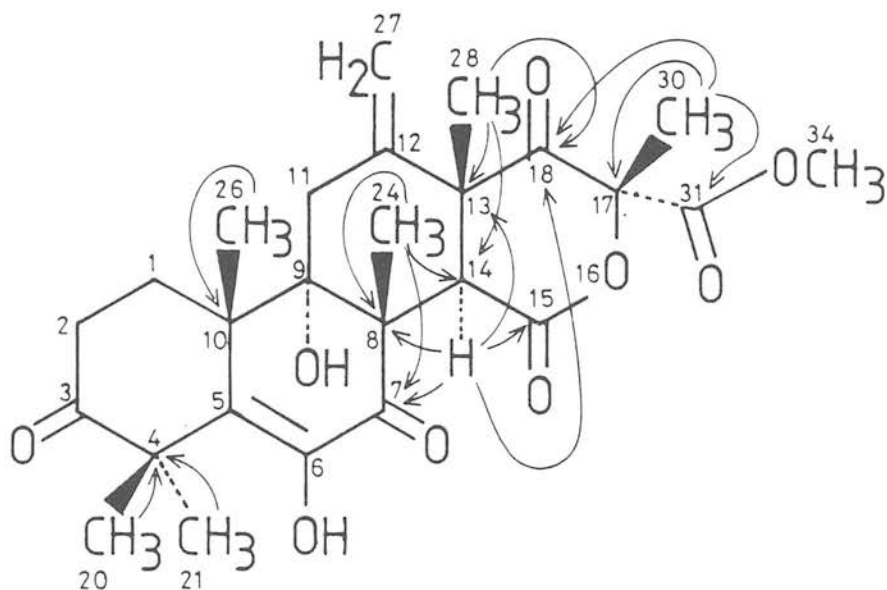


Figure 9 (contd...)

Otherwise, only four unfunctionalised quaternary positions; C-4, 8, 10 and 13, remained to be assigned. As the only decoupling to even slightly affect the carbon signal at 43.0 p.p.m. was that of H-26, which caused a barely perceptible increase in relative intensity, this signal must be due to C-10 which is isolated from the other decoupled protons. The paucity of effect of decoupling H-26 is surprising; this is perhaps due to the presence of the seven other protons within 3 bond lengths of C-10 which could conceivably couple to it. However, the C-10 signal is not particularly broad, suggesting that C-10 is not coupled to an unusual extent.

The signal at 52.2 p.p.m. sharpened from a multiplet to a triplet on decoupling H-24, and an intensity increase was also noted when the H-14 proton was decoupled. This signal can thus be allocated to C-8.

When either the H-28 or the H-20/21 proton frequencies were irradiated, the carbon resonance at 47.7 p.p.m. sharpened very markedly. As no carbon is within coupling or n.O.e. range of both the H-28 protons and the protons of the gem-dimethyl group, this effect is attributed to insufficiently selective irradiation of the proton frequencies. The sharpening was more pronounced for the H-20/21 decoupling suggesting that C-4 is responsible for this signal. This was supported by the response of the remaining quaternary signal at 49.3 p.p.m. to decoupling. Although here again the decouplings of H-28 and H-20/21 both produced a



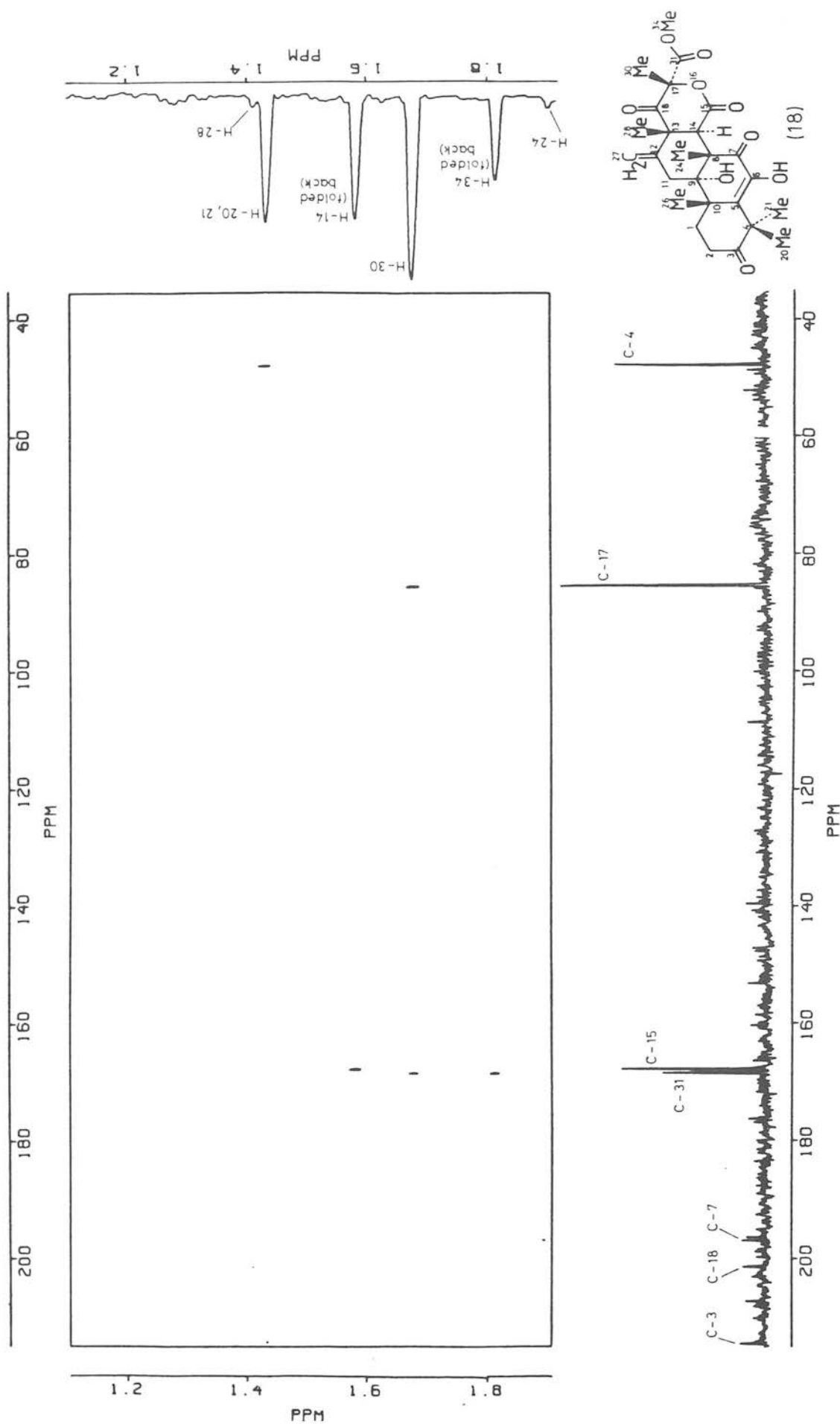


Figure 11 Two-dimensional heteroscalar correlation n.m.r. spectrum (360 MHz and 90.6 MHz) of terretinin (in  $\text{CDCl}_3$ ) identifying long-range couplings between the methyl protons and  $^{13}\text{C}$  nuclei more than one bond removed

discernable sharpening, the effect this time was more pronounced for H-28. In addition, an intensity increase was observed on decoupling H-14; hence this signal is probably due to C-13.

Rather less equivocal results for C-4 were obtained from a second two-dimensional, carbon-hydrogen plot (Figure 11), this time designed<sup>37</sup> to show the correlations between the methyl protons and the carbons more than one bond removed. The interactions thus revealed are shown in Figure 12. A clear correlation was seen between the carbon signal at 47.7 p.p.m. and the proton signal at 1.44 p.p.m. due to H-20/21, confirming the assignment of the carbon signal to C-4. These protons were also shown to correlate to the signal at 213.9 p.p.m., reinforcing its assignment to C-3 previously based only on elimination of the other two contenders. The H-28 proton signal at 1.41 p.p.m. only showed one correlation (to C-18 at 201.2 p.p.m.) in this plot. No unexpected correlations were present in this two-dimensional spectrum although two signals were observed, at carbon frequencies 167.6 p.p.m. (C-15) and 168.3 p.p.m. (C-31), which did not correspond to any of the methyl proton frequencies. These are probably due to "fold-back" of the H-14 and H-34 resonances which were outside the proton range of this spectrum.

The complete list of  $^{13}\text{C}$  assignments is given in Table 3 (Section 4.3.5). C-5 and C-12 were distinguished on the basis of  $^1\text{J}_{\text{CC}}$  coupling data.

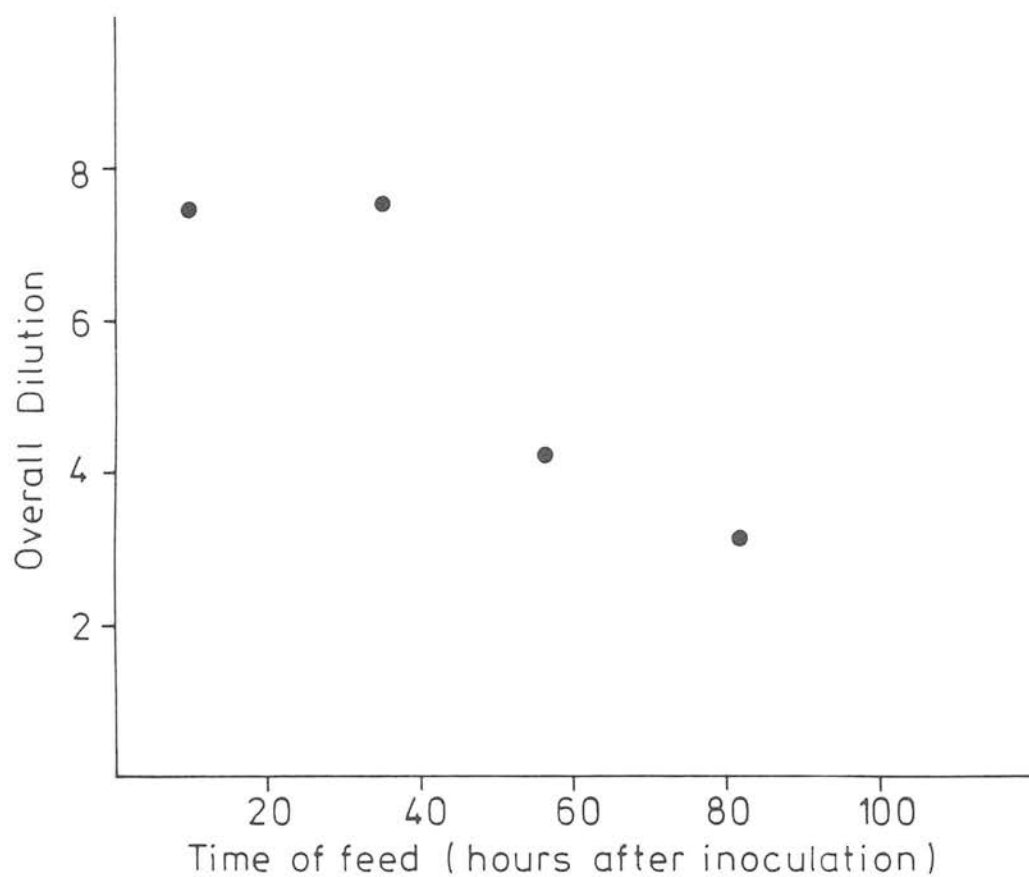


Figure 13 Apparent dilutions of  $[2-^{14}\text{C}]$ acetate in terretonin  
(but see text)

#### 4.3.4 Incorporation of [2-<sup>14</sup>C]acetate

In order to establish the optimum time to feed <sup>13</sup>C-labelled sodium acetates, preliminary experiments with radiolabelled acetate were performed. Samples of [2-<sup>14</sup>C]-acetate were administered to cultures of *A. terreus* at four different times after inoculation; terretonin was isolated and purified to constant activity. Because of the difficulty experienced in crystallising terretonin, this purification was carried out by repetitive t.l.c., a procedure which subsequently came under suspicion.

Nevertheless, the results as found are depicted in Figure 13. They indicated a sharp drop in the specific dilution of <sup>14</sup>C acetate given more than two days after inoculation. The 81.5 hour feed resulted in an apparent specific dilution of 3.1 fold. For a sesquiterpene-tetraketide biosynthesis, an average dilution of 13 x 3.1 fold per labelled site was indicated. An average <sup>13</sup>C enrichment of 2.5% would therefore be expected if 100% <sup>13</sup>C-labelled acetate was fed at this time.

#### 4.3.5 Incorporation of <sup>13</sup>C-Labelled Acetates and Methionine

With this encouragement, [1-<sup>13</sup>C]- and [1,2-<sup>13</sup>C<sub>2</sub>]acetates and [Me-<sup>13</sup>C]methionine were administered to different 83.5 hour cultures of *A. terreus*. The three terretonin samples subsequently isolated were each examined by p.n.d. <sup>13</sup>C n.m.r. spectroscopy. Scrutiny of the spectrum from the sample supposedly labelled by [1-<sup>13</sup>C]acetate, however, revealed negligible enrichment above natural abundance.

Table 3 Summary of  $^{13}\text{C}$  n.m.r. assignments and the results of  $^{13}\text{C}$  incorporation experiments on terretonin

Carbon	$\delta_{\text{C}}$ (p.p.m.) <sup>a</sup>	$^1J_{\text{CC}}$ (Hz) <sup>b</sup>	Carbon	$\delta_{\text{C}}$ (p.p.m.) <sup>a</sup>	$^1J_{\text{CC}}$ (Hz) <sup>b</sup>
1	27.9		14	44.4	55
2	32.5	38	15	167.6	55
3	213.9	38	17	85.3	61
4	47.7	37	18	201.2	-
5	131.5	84	20 <sup>c</sup>	21.1	37
6	138.5	84	21 <sup>c</sup>	23.5	-
7	196.8	-	24	19.6	33
8	52.2	34	26	18.4	34
9	77.5	35	◆ 27	116.7	
10	43.0	34	28	23.3	32
11	34.7	35	◆ 30	21.1	
12	139.6	-	31	168.3	61
13	49.3	32	◆ 34	53.5	

◆ labelled by  $[\text{Me-}^{13}\text{C}]\text{methionine}$

<sup>a</sup> In  $\text{CDCl}_3$

<sup>b</sup> From incorporation of  $[1,2\text{-}^{13}\text{C}_2]\text{acetate}$

<sup>c</sup> May be interchanged

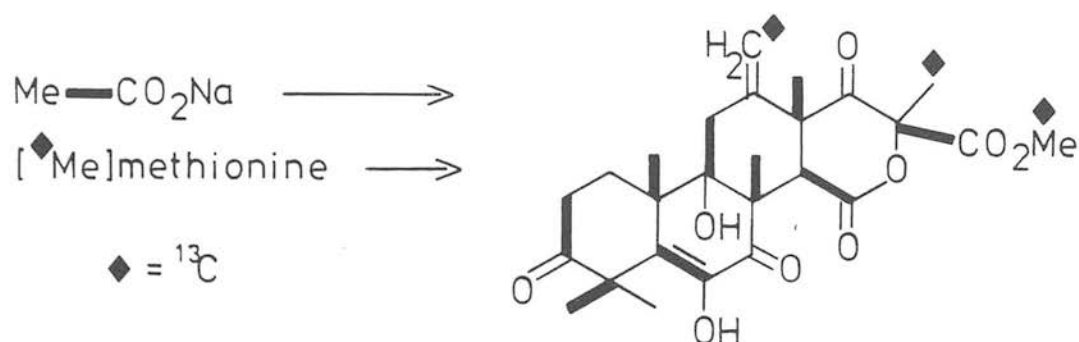
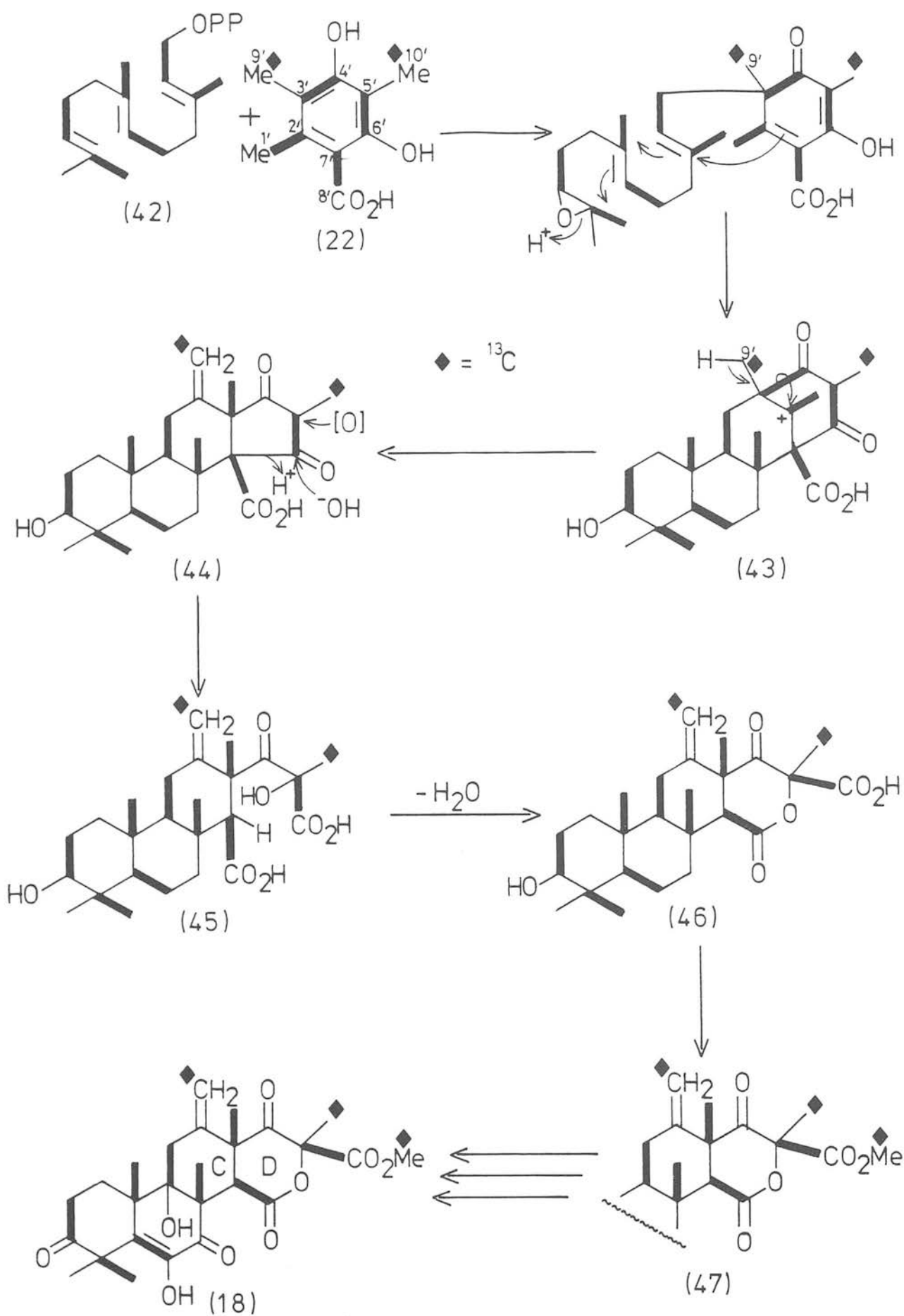


Figure 14 Labelling of terretonin by  $[1,2\text{-}^{13}\text{C}_2]\text{acetate}$  and by  $[\text{Me-}^{13}\text{C}]\text{methionine}$



Fortunately,  $^{13}\text{C}$ - $^{13}\text{C}$  coupling satellites were visible in the spectrum of terretonin labelled from  $[1,2-^{13}\text{C}_2]$ -acetate although they were very small. The average level of enrichment, based on the relative height of the satellite and centre lines, was estimated at around 0.3% which is close to the practical limit of the double  $^{13}\text{C}$ -label technique without recourse to simultaneous depletion of natural abundance  $^{13}\text{C}$ . However, the coupling constants were determined and the results are summarised in Table 3. These, incidentally, allow the assignment of the C-5 and C-12 resonances: as the signal at 131.5 p.p.m. shows a  $^1\text{J}(^{13}\text{C}-^{13}\text{C})$  coupling of 84 Hz, equal to that of the C-6 resonance, this signal must be due to C-5. The signal at 139.6 p.p.m. shows no coupling, consistent with its assignment to C-12.

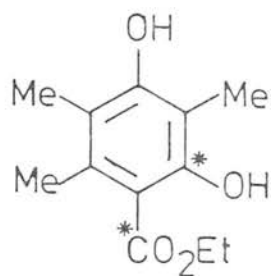
The methionine-labelled sample showed three very enhanced spectral lines at 21.1, 53.4 and 116.7 p.p.m. For each line, comparison with a natural abundance spectrum indicated a  $^{13}\text{C}$  enrichment of over 5%. This sample also exhibited a very intense signal at 42.5 p.p.m. which did not correspond to any of the carbon signals of terretonin. Attempts to remove this impurity by t.l.c. failed. The size of this anomalous signal, from what otherwise appeared to be a reasonably pure sample of terretonin, suggested that it was perhaps due to some derivative of the labelled methionine. It cannot be due to methionine itself, the *S*-methyl  $^{13}\text{C}$  resonance of which appears at around 15 p.p.m.<sup>38</sup>



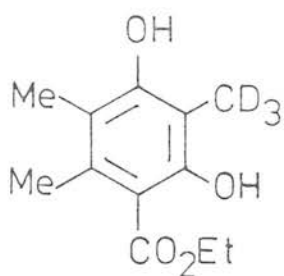
Scheme 11

The other disturbing aspect of these experiments was the low incorporation of the  $^{13}\text{C}$ -labelled acetates compared with the predictions of the radiolabelling study. Such a large difference can only reasonably be ascribed to failure to secure radiochemically pure material and indicates that some minor impurity of relatively high activity had not been removed by t.l.c.

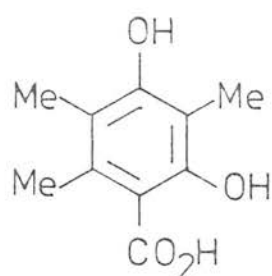
Despite this, sufficient information was gained (Table 3) to support the hypothesis of a sesquiterpene-tetraketide biosynthesis. A labelling pattern for terretonin (18) as illustrated in Figure 14 was indicated. This is consistent with the formation of terretonin (Scheme 11) from the same intermediates, farnesyl pyrophosphate (42) and dimethylorsellinic acid (22), implicated in the biosynthesis of the *A. variegator* metabolites and austin (17). Cyclisation in the same general manner as austin would generate the incipient carbocation (43). If this was quenched by ring-contraction and loss of a proton from the methionine-derived 9'-methyl group instead of, as in austin, from the acetate-derived 1'-methyl group, the cyclopentadione (44) would be formed. A retro-Claisen type reaction and the introduction of a hydroxyl group to give the diacid (45) followed by condensation to the lactone (46) and methylation by *S*-adenosyl methionine would give the correct structure (47) of the C and D rings of terretonin (18) with the labelling pattern indicated by the  $^{13}\text{C}$  experiments. The labelling of the rest of the molecule would follow from a cyclisation of the farnesyl group as shown.



(12)

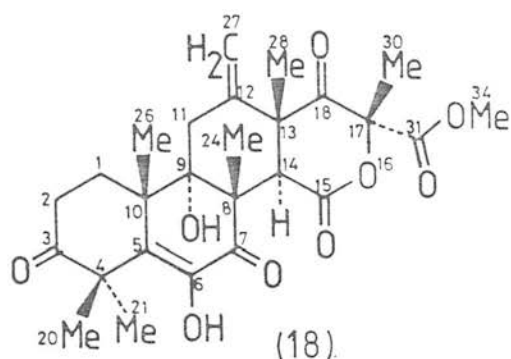


(13)



(22)

\* =  $^{14}\text{C}$



(18)

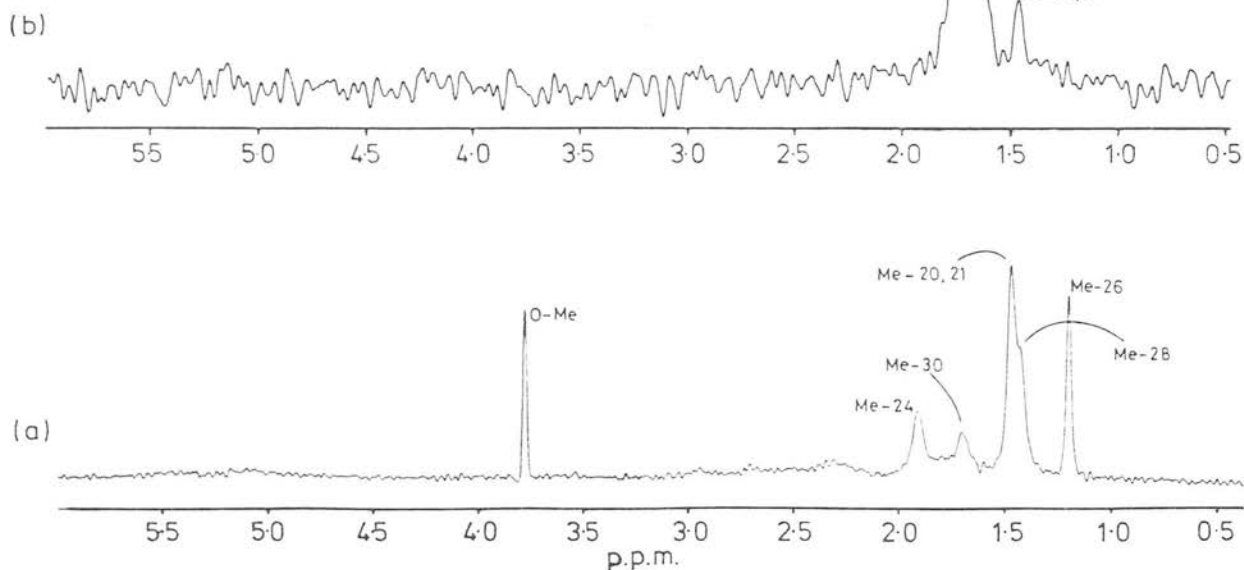


Figure 15 55.3 MHz  $^2\text{H}$  n.m.r. spectra of terretonin (in  $\text{CHCl}_3$ ):  
 (a) labelled by  $\text{D}_2\text{O}$ ; (b) labelled by ethyl [3-methyl,  
 $^2\text{H}_3$ ]-3,5-dimethylorsellinate

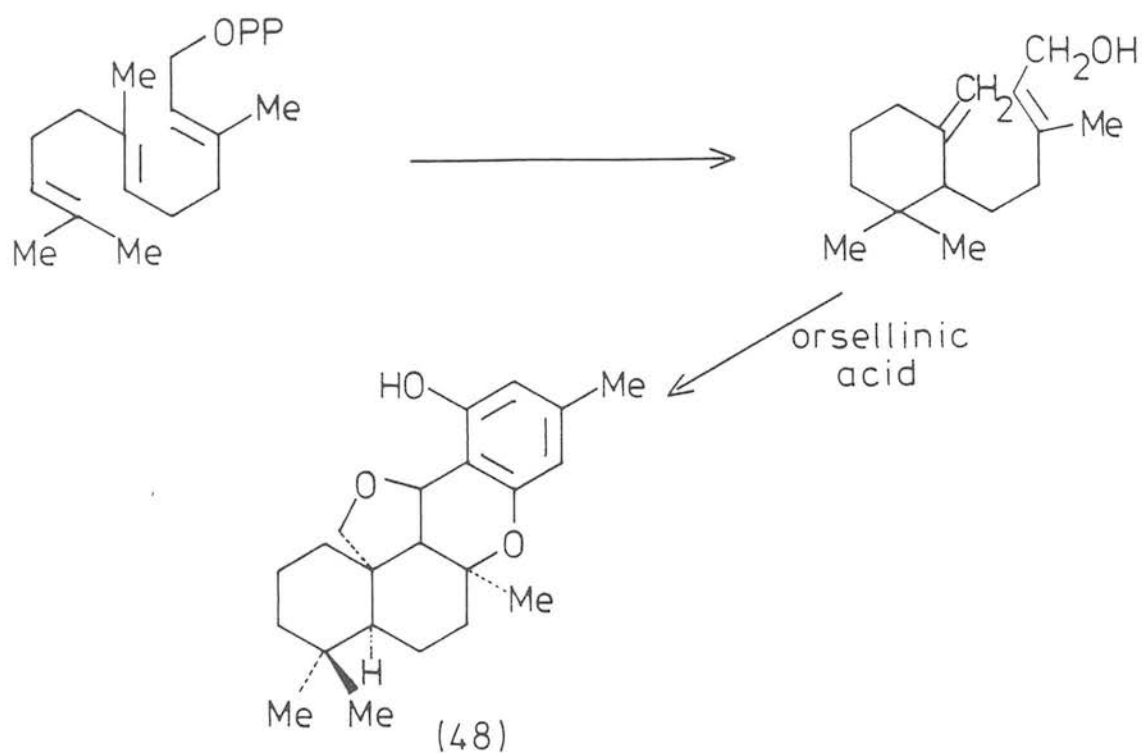
#### 4.3.6 Incorporation of Labelled Orsellinates

As in the case of austin, the proposed pathway requires a great deal of modification to the orsellinate moiety which was so far unproven as an intermediate. In order to test this point, *A. terreus* was grown in the presence of ethyl [carboxyl, 2- $^{14}\text{C}$ ]-3,5-dimethylorsellinate (12)<sup>39</sup> and terretonin was subsequently isolated. Again, the reluctance of terretonin to recrystallise presented difficulties. After the normal isolation procedure had been completed and scintillation counting had shown a specific dilution of 39.5, a different t.l.c. solvent, which had previously been observed to resolve terretonin from a trace amount of slightly more polar material, was used. The dilution was now 38.2, *i.e.* the activity of the sample had, if anything, increased. This gives a specific incorporation of *ca.* 2.5%. To check that this result was not spurious and to ensure that any  $^{14}\text{C}$ -labelling was from the intact incorporation of dimethylorsellinate and not due to degradation to, and reincorporation of, acetate units, ethyl [3-methyl,  $^2\text{H}_3$ ]-3,5-dimethylorsellinate (13)<sup>39</sup> was administered to *A. terreus* cultures in a separate experiment.

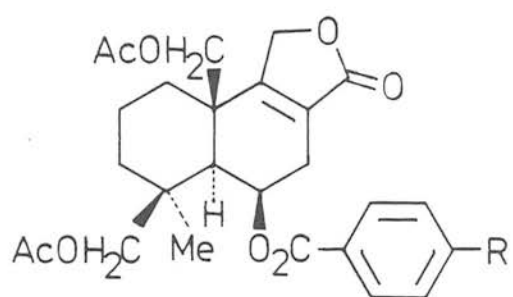
Terretonin thus derived was examined by  $^2\text{H}$  n.m.r. spectroscopy. The spectrum was compared with that of a sample of terretonin produced by growing *A. terreus* in a medium in which 5% of the water had been replaced by  $\text{D}_2\text{O}$  (Figure 15). (This useful method of obtaining a metabolite

generally enriched with deuterium was used in a biosynthetic study<sup>40</sup> of aflatoxin B<sub>1</sub>.) The methyl signals in the <sup>2</sup>H spectra are assigned by comparison with the proton spectrum. It is apparent that the only position significantly labelled by the specifically deuterated orsellinate is that of the C-30 methyl group (1.68 p.p.m. in <sup>1</sup>H and <sup>2</sup>H n.m.r. spectra) in accordance with Scheme 11. A small signal at 1.45 p.p.m., which corresponds to the C-20 and C-21 methyls, is also visible and suggests that perhaps a small degree of degradation and reincorporation has occurred. It is too large to be due to natural abundance unless the incorporation of the orsellinate is much lower than that indicated by the corresponding <sup>14</sup>C experiment. This experiment shows, therefore, that ethyl 3,5-dimethyl-orsellinate (12) can act as a specific precursor to terretonin, although the free acid (22) is more likely to be the true intermediate. Good evidence for the meroterpenoid pathway depicted in Scheme 11 is thus provided.

The <sup>2</sup>H n.m.r. spectrum of terretonin produced in the presence of D<sub>2</sub>O is useful as it shows that sufficient resolution of the methyl resonances is possible for an unambiguous assignment of the C-30 methyl deuterium signal. However, this spectrum also contained some puzzling features. The deuterium labelling did not appear to be uniform by any means: the signals from the exocyclic methylene deuterons were extremely weak and that expected from the C-14 methine hydrogen position was absent altogether. According to the proposed biosynthesis,



Scheme 12



(49) R = H

(50) R = OH

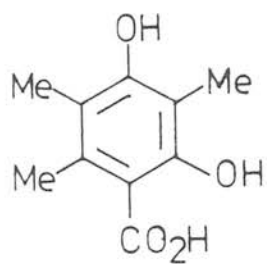
H-14 would be derived from the medium *via* a retro-Claisen type reaction and therefore would necessarily be labelled. Yet it apparently carries no deuterium whereas positions derived from acetate methyls do. Possibly any methine deuterium was exchanged out during work-up.

#### 4.3.7 Conclusions

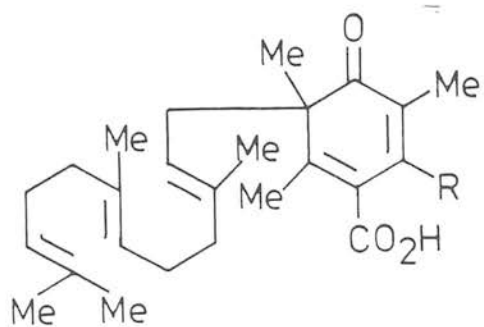
On the evidence of these experiments, terretonin appears to belong to the triprenyl-phenol group of fungal metabolites and to share a biosynthetic pathway common, at least in its early stages, to the andibenins, andilesins, anditomin and austin. As in the cases of the two last-named compounds, oxidative modification of the orsellinate-derived carbocyclic ring involves a cleavage of the C-3',4' bond. The bicyclofarnesyl part of the molecule, however, suffers no further skeletal rearrangements after cyclisation, although it is extensively oxidised. Although the absolute stereochemistry of terretonin has not been determined, it is noteworthy that the stereochemistry at C-9 relative to C-10 of terretonin is the opposite of that at C-9 of andilesin. Perhaps this has an influence on further modifications of the skeleton.

It is also conceivable that the stereochemistry of the cyclisation of the farnesyl group reflects the stage at which it occurs. Cell-free studies<sup>41</sup> on the biosynthesis of the triprenyl-phenol, siccanin (48), from *Helminthosporium siccanis*, provided circumstantial evidence for cyclisation of

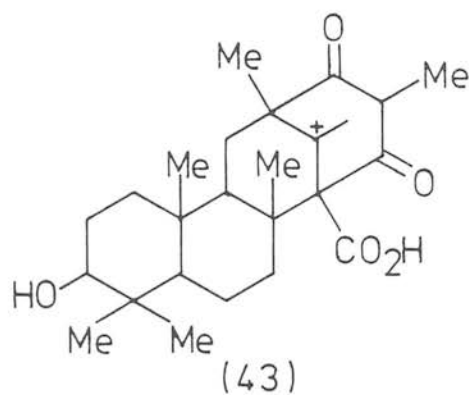




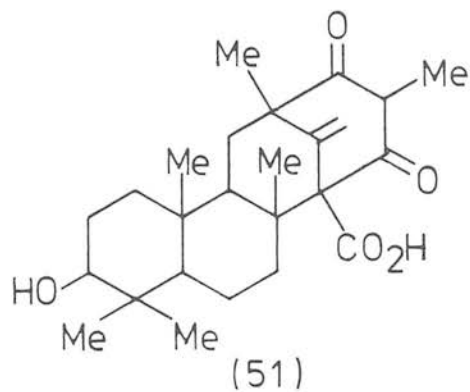
(22)



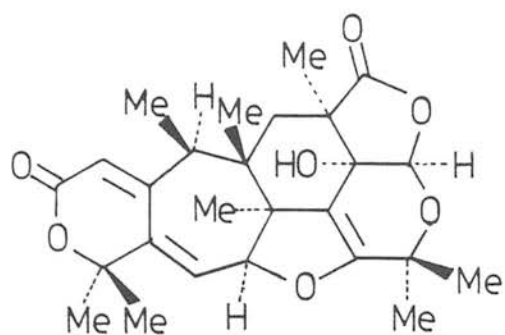
(8) R = H



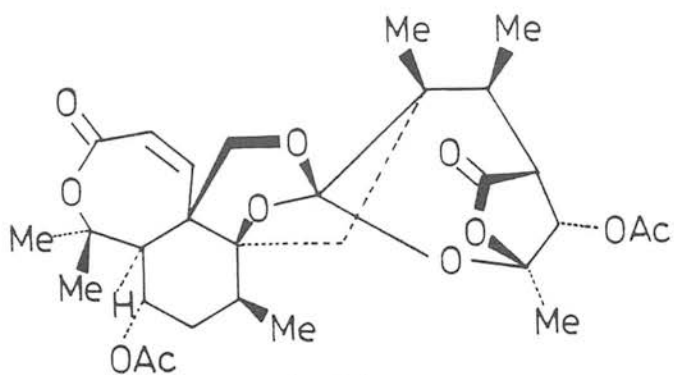
(43)



(51)



(52)



(53)

farnesol occurring, at least in part, before the alkylation of orsellinic acid (Scheme 12). In addition, an *A. variegator* mutant, impaired in polyketide production, has been observed<sup>42</sup> to produce cyclic sesquiterpenes in the form of astellolides A (49) and B (50). It would be interesting to discover whether the uncyclised adduct (8) is a true intermediate of the *Aspergillus* meroterpenoids.

Later intermediates, also, of the pathway depicted in Scheme 11 are not as yet well characterised. Further experiments with <sup>2</sup>H- and <sup>18</sup>O-labelled precursors could yield information in this respect. In particular, it would be interesting to establish how many acetate-derived hydrogens are retained by the C-28 methyl group of terretonin. This is originally the "starter" methyl of the orsellinate (22) intermediate and would be expected to retain up to three such hydrogens if the carbocation species (43) is an intermediate. However, if the exocyclic methylene species (51) proposed for the biosynthesis of austin is involved, no more than two acetate hydrogens could remain.

Recent observations have extended the range of tri-prenyl-phenol fungal metabolites<sup>43</sup>. Of particular interest in this context are two recently reported compounds: paraherquonin<sup>44</sup> (52) from *Penicillium paraherquei* and fumigatonin<sup>45</sup> (53) from *Aspergillus fumigatus*. Biosyntheses from farnesyl pyrophosphate and 3,5-dimethylorsellinate were postulated in both cases. Fumigatonin would only have one carbon linkage between the terpenoid and tetra-

ketide moieties although the points of attachment would be the same as in the proposed intermediate (8) of andibenin *etc.* Structural analysis of paraherquonin suggests that it is most closely related to austin (17) although the degree of oxidative modification required to give the final structure (52) is exceptional.

#### 4.4 Experimental

(General procedures and instrumentation are described in Section 3.6.1).

##### 4.4.1 Andibenin B/Andilesin A

*Production and Isolation of andibenin B and andilesin A*  
*Aspergillus variegator* - strain 212I69 (a u.v. induced mutant strain derived from NRRL 212) was stored and subsequently grown in exactly the same manner as the xanthone-producing strain (see Chapter 5) except that the period of growth on malt-extract broth was only 8-10 days.

Work-up proceeded by decantation of the medium from the mycelium and filtration to remove any fine debris. The liquor was acidified, with 2N aqueous HCl, from the harvest pH of *ca.* 7 to pH 2, and extracted with ethyl acetate (4 x one-half of liquor volume). After drying ( $\text{MgSO}_4$ ) the extract, the solvent was removed *in vacuo* to give a brown gum (about  $400 \text{ mg l}^{-1}$ ), the constituents of which were separated by preparative t.l.c., developing with methanol-chloroform (4:96). The band at  $R_f = 0.5$ , due to both andibenin B and andilesin A, was eluted with ethyl acetate to yield a white solid ( $100\text{-}150 \text{ mg l}^{-1}$ ). Further t.l.c., developing twice with ethyl acetate-petroleum ether (b.p.  $40\text{-}60^\circ\text{C}$ ) (50:50), effected separation. The andibenin B band ( $R_f = 0.4$ ) was readily visible under u.v. light, but that due to andilesin A was not; however, elution of the whole area below the andibenin B band and above the base-line proved satisfactory. Andibenin B was thus isolated

as a white crystalline solid (about 100 mg l<sup>-1</sup>) which recrystallised from methanol to give square prisms, m.p. 218-223°C (lit.<sup>3</sup>, 219-220°C). Andilesin A was isolated as long white needles (15-30 mg l<sup>-1</sup>), which, after recrystallisation from ethyl acetate, had m.p. above 308°C (sub. dec.) (lit.<sup>3</sup>, 310°C ). In each case spectral and physical data (m.s., <sup>1</sup>H n.m.r., <sup>13</sup>C n.m.r., t.l.c.) were in agreement with those of authentic samples and with published<sup>3,4</sup> values.

*Growth-Production study of andibenin B-andilesin A*

*Aspergillus varicolor* - strain 212I69 - was grown as previously described. At various times after inoculation, two of the culture flasks were worked up. The weight of the andibenin B-andilesin A mixture was recorded in each case.

Results

Time/hours	Extract Weight/g l <sup>-1</sup>	Andibenin B plus Andilesin A/mg l <sup>-1</sup>
68	0.29	35
95	0.35	30
118	0.37	50
142	0.60	95
163	0.57	90
235	-	110
259	-	55

*Incorporation of sodium [1-<sup>14</sup>C]acetate into andibenin B*

*Aspergillus variegatus* - strain 212I69 - was grown as previously described in 31 flasks. Thirteen hours after inoculation, a sterile solution of sodium [1-<sup>14</sup>C]acetate (500 mg, 11.1  $\mu$ Ci, Sp.Act. =  $4.04 \times 10^6$  dpm mmol<sup>-1</sup>) in distilled water (20 ml) was distributed evenly by syringe into the culture media of five flasks. Parallel experiments, feeding at 25, 36 and 50 hours, were conducted concurrently. A further, identical such dose of acetate was made up to 40 ml and, at each of the aforementioned times, one quarter of the solution was distributed evenly among the same five flasks ("pulsed feed" experiment). A hypodermic needle was used to inject the solutions through the mycelial mat. Six flasks were retained as controls. After a total incubation time of 201 hours, the flasks were refrigerated and the andibenin B was isolated from each batch.

Results

Time fed/ hours	Yield/mg l <sup>-1</sup>	Sp.Act./dpm mmol <sup>-1</sup>	Dilution
(control)	98.8		
Pulse fed	59.2	$2.59 \times 10^5$	15.6
13	62.8	$2.08 \times 10^5$	19.4
25	82.6	$2.16 \times 10^5$	18.7
36	72.0	$3.16 \times 10^5$	12.8
50	43.6	$5.25 \times 10^5$	7.7

*Incorporation of sodium [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate into andibenin B and andilesin A*

*Aspergillus variegator* - strain 212I69 - was grown as previously described in 12 flasks. Fifty hours after inoculation, a sterile solution of sodium [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]-acetate (1.000 g, 90 atom % <sup>13</sup>C, 81 atom % <sup>18</sup>O<sub>2</sub>, 18 atom % <sup>18</sup>O<sub>1</sub>) in distilled water (40 ml) was distributed evenly by syringe among the culture media of 10 flasks. A hypodermic needle was used to inject the solution through the mycelial mat. Two flasks were retained as controls. As growth progressed, the media of the acetate-fed cultures became significantly darker in colour than those of the control cultures. At 245 hours, when the cultures were worked up, the controls appeared healthy whereas the labelled cultures appeared to be dying.

Results

	Yield of andibenin B/mg l <sup>-1</sup>	Yield of andilesin A/mg l <sup>-1</sup>
Acetate fed	97	29
Control	105	30

*Incorporation of <sup>18</sup>O<sub>2</sub> into andibenin B and andilesin A*

*Aspergillus variegator* - strain 212I69 - was grown as previously described in 16 flasks. Four were fitted with foam rubber bungs as usual, and retained as controls. The other 12 were attached to the "closed volume" apparatus

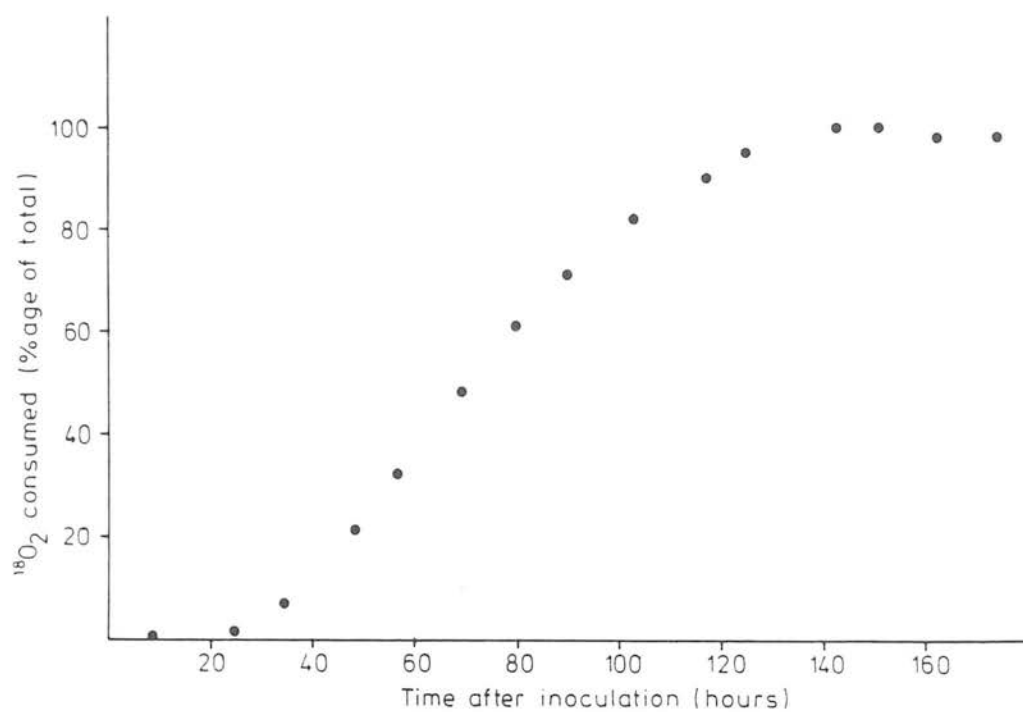


Figure 16 Uptake of  $\text{O}_2$  gas by cultures of *Aspergillus variegatus* 212I69



(Appendix ) for measuring the uptake of oxygen (see Figure 16), and grown under an atmosphere composed initially of  $^{18}\text{O}_2$ - $^{14}\text{N}_2$  (30:70). Any oxygen deficit was replaced by further  $^{18}\text{O}_2$  at the times of observation. After 174 hours of incubation, the andibenin B and andilesin A were isolated.

### Results

Yield of $^{18}\text{O}_2$ -labelled andibenin B	59 mg l <sup>-1</sup>
Yield of $^{18}\text{O}_2$ -labelled andilesin A	17 mg l <sup>-1</sup>

This strain was known also to produce tajixanthone (see Chapter 5), in small quantities, isolable from the mycelium, so the dried mycelium of both control and experimental cultures was extracted by Soxhlet apparatus with petroleum ether (b.p. 40-60°C). Surprisingly, however, only the control extracts showed the presence of tajixanthone, as revealed by its deep yellow colour and by analytical t.l.c.

#### 4.4.2 Terretonin

##### *Production and isolation of terretonin*

*Aspergillus terreus* NRRL 6273, was stored in the dark at 4°C under liquid paraffin on slopes of potato dextrose agar (Oxoid CM 139). Cultures for large scale inoculations were prepared from these by growth, also on potato dextrose agar, in medical flats at 26°C in constant light for 8-10 days. A spore suspension in distilled water was subsequently used to inoculate the production medium of malt-extract broth (Oxoid Malt-Extract, 3% w/v; Oxoid Mycological

Peptone, 0.5% w/v; distilled water, to 100%) 200 ml of which was contained in each of the penicillin flasks employed. The fungus was allowed to grow for 14 days in constant light at 26°C. A thick, brown, mycelial mat formed on the surface of the medium.

The growth medium was carefully decanted from the flasks and filtered through muslin to remove any stray pieces of mycelium. A little hot water, used to rinse the flasks and mycelium, was also filtered and added to the medium. The liquor was then extracted, at the harvest pH of *ca.* 7, with chloroform (4 x one-third of liquor volume): the extract was dried ( $\text{MgSO}_4$ ) and reduced *in vacuo* to give a brown foam (typically  $150 \text{ mg l}^{-1}$ ). This was subjected to preparative t.l.c., developing with acetone-chloroform (14:86), using a very light loading (20 mg per plate). Elution (chloroform) of the band at  $R_f = 0.4$  gave terretonin ( $30\text{--}35 \text{ mg l}^{-1}$ ) appearing as a white foam on careful evaporation of the solvent. Recrystallisation (difficult) from ethyl acetate gave, in small yield, white, many-sided prisms, m.p.  $261.5 - 264.5^\circ\text{C}$  (sub., dec.) lit.<sup>13</sup>,  $260\text{--}262^\circ\text{C}$ ). Spectral data were in agreement with published<sup>13</sup> values.

*Incorporation of sodium [2-<sup>14</sup>C]acetate into terretonin*

*Aspergillus terreus* was grown, as previously described, in 25 penicillin flasks. Nine hours after inoculation, a sterile solution of sodium [2-<sup>14</sup>C]acetate (1.000 g, 10  $\mu$ Ci, Sp.Act.  $1.82 \times 10^6$  dpm mmol<sup>-1</sup>) in distilled water (20 ml) was distributed evenly by syringe into the culture media of 5 flasks. Parallel experiments, feeding at 34.5, 56 and 81.5 hours, were conducted concurrently. Care was taken not to disturb the mycelial mat when feeding: a hypodermic needle was used to deliver the solution below the surface. Five flasks were retained as controls. All the flasks were incubated for a total of 338 hours. Terretonin was isolated and purified as usual.

Results

Experiment	Yield of terretonin/mg l <sup>-1</sup>	Specific Activity /dpm mmol <sup>-1</sup>	Dilution
Control	36.0		
9 hour feed	27.6	$2.45 \times 10^5$	7.4
34.5 hour feed	28.3	$2.43 \times 10^5$	7.5
56 hour feed	29.1	$4.38 \times 10^5$	4.2
81.5 hour feed	30.5	$5.89 \times 10^5$	3.1

*Incorporation of [1-<sup>13</sup>C]-and [1,2-<sup>13</sup>C<sub>2</sub>]acetates and [methyl-<sup>13</sup>C]-L-methionine into terretonin*

*Aspergillus terreus* was grown, as previously described, in 15 penicillin flasks. 83.5 hours after inoculation, a sterile solution of sodium [1-<sup>13</sup>C]acetate (1.000 g, 90.6 atom % <sup>13</sup>C) in distilled water (20 ml) was distributed evenly into the culture media of five flasks by injection through the mycelial mat. Parallel experiments, feeding with 20 ml solutions of sodium [1,2-<sup>13</sup>C<sub>2</sub>]acetate (1.000 g, 90.0 atom % <sup>13</sup>C) and [methyl-<sup>13</sup>C]-L-methionine (200 mg, 90.0 atom % <sup>13</sup>C), also at 83.5 hours, were conducted concurrently. All the flasks were incubated for a total of 336 hours.

Results

Experiment	Yield of terretonin/mg l <sup>-1</sup>
[1- <sup>13</sup> C]acetate	31
[1,2- <sup>13</sup> C <sub>2</sub> ]acetate	33
[Me- <sup>13</sup> C]-L-methionine	30

*Incorporation of ethyl [carboxyl, 2-<sup>14</sup>C]-2,4-dihydroxy-3,5,6-trimethylbenzoate (12) into terretonin*

Ethyl [carboxyl, 2-<sup>14</sup>C]-2,4-dihydroxy-3,5,6-trimethylbenzoate<sup>39</sup> (12) (33 mg, 39.7 μCi mmol<sup>-1</sup>) was dissolved in hot distilled water (25 ml) also containing "Tween 80" detergent (0.5 ml). The sterilized solution was distributed evenly, by injection through the mycelial mat, into the culture media of

five penicillin flasks, each containing a 73 hour culture of *Aspergillus terreus*, grown as previously described. After a total of 335 hours growth, terretonin was isolated as normal. On this occasion, however, extra purification was effected by further preparative t.l.c., developing with ethyl acetate-methylene chloride (25:75), which separated terretonin ( $R_f = 0.49$ ) from a slightly more polar impurity present only in trace quantity.

### Results

Yield of terretonin	33.8 mg l <sup>-1</sup>
Specific activity	$2.31 \times 10^6$ dpm mmol <sup>-1</sup>
Percentage incorporation	1.2
Dilution	38

#### *Incorporation of ethyl [3-methyl-<sup>2</sup>H<sub>3</sub>]-2,4-dihydroxy-3,5,6-trimethylbenzoate (13) into terretonin*

A sterile solution of ethyl [3-methyl-<sup>2</sup>H<sub>3</sub>]-2,4-dihydroxy-3,5,6-trimethylbenzoate<sup>39</sup> (13) (50 mg) in distilled water (50 ml), also containing "Tween 80" detergent (0.5 ml) was fed to 5 flasks of a 73 hour culture of *Aspergillus terreus* exactly as described above. After 336 hours, terretonin (35 mg) was isolated as usual. Further purification, effected by preparative t.l.c., developing with ethyl acetate-methylene chloride (25:75), yielded terretonin (27 mg).

*Preparation of generally deuterated terretonin*

*Aspergillus terreus* was grown, as previously described, in 10 penicillin flasks. In five of these, 5% of the water had been substituted by D<sub>2</sub>O. The flasks were incubated for 336 hours and the terretonin was isolated as usual.

Results

	Yield of terretonin/mg l <sup>-1</sup>
Control	39
D <sub>2</sub> O	37

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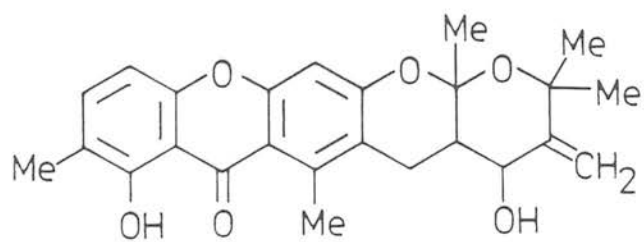


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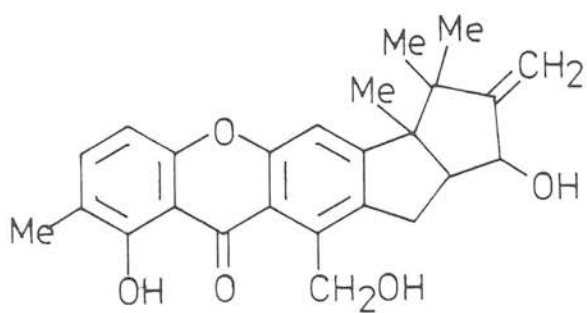
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Chapter 5

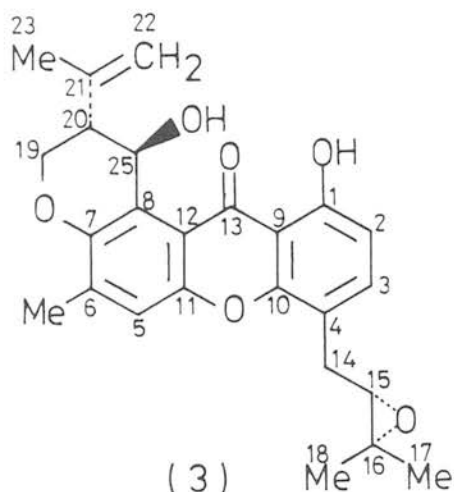
Biosynthetic Studies of Tajixanthone  
and Shamixanthone



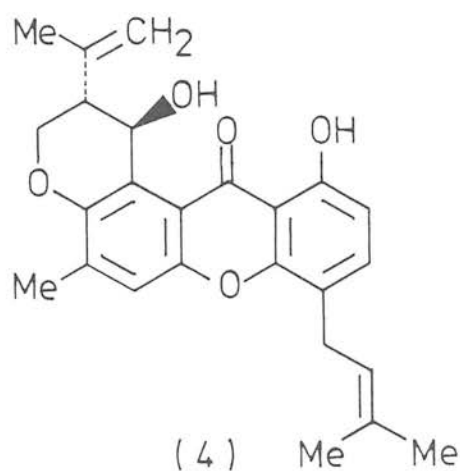
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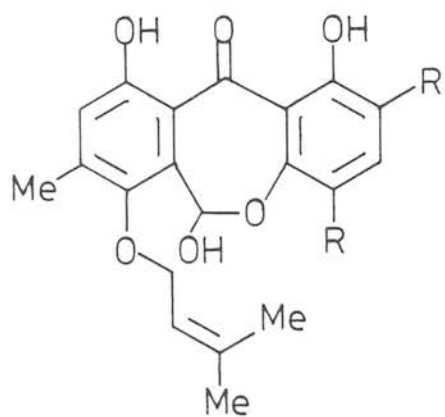
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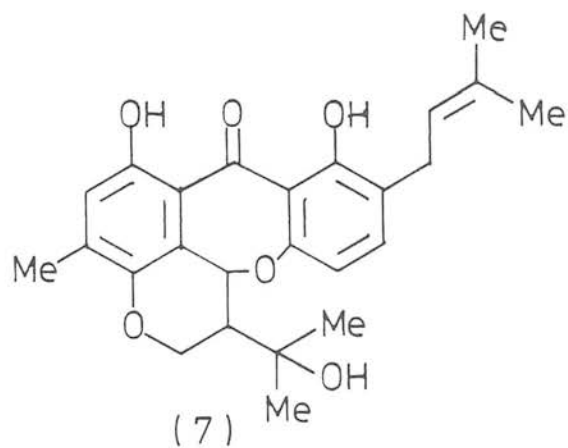


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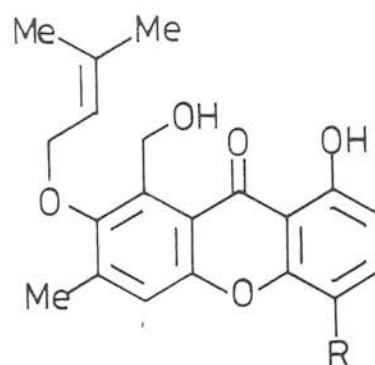


(5)  $R^1 = \text{CH}_2\text{CH}:\text{CMe}_2$ ,  $R^2 = \text{H}$

(6)  $R^1 = \text{H}$ ,  $R^2 = \text{CH}_2\text{CH}:\text{CMe}_2$



(7)



(8)  $R = \text{H}$

(9)  $R = \text{CH}_2\text{CH}:\text{CMe}_2$

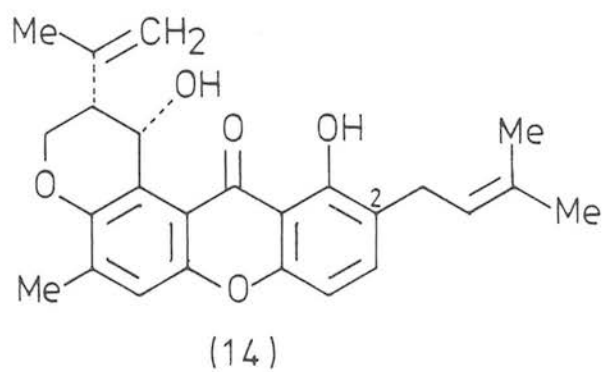
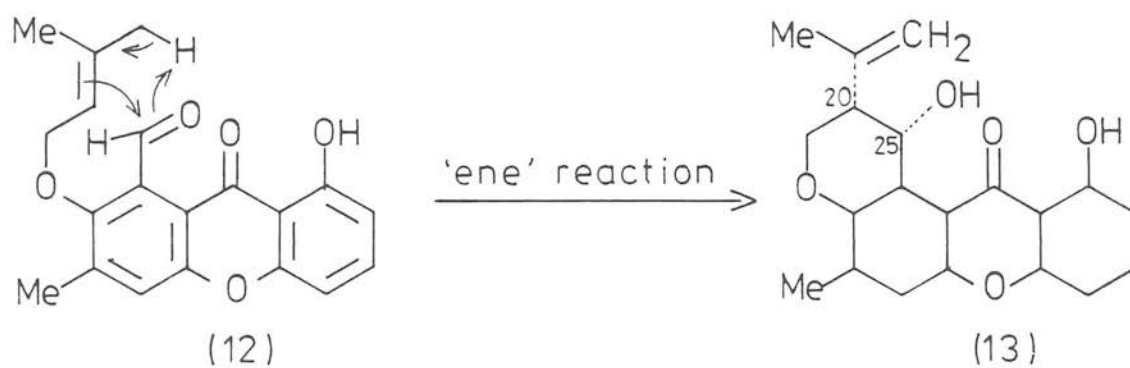
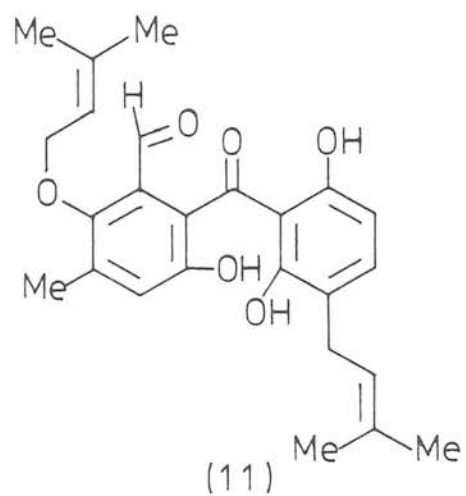
(10)  $R = \text{CH}_2\text{CH}(\text{CMe}_2)\text{O}$

## 5 Biosynthetic Studies of Tajixanthone and Shamixanthone

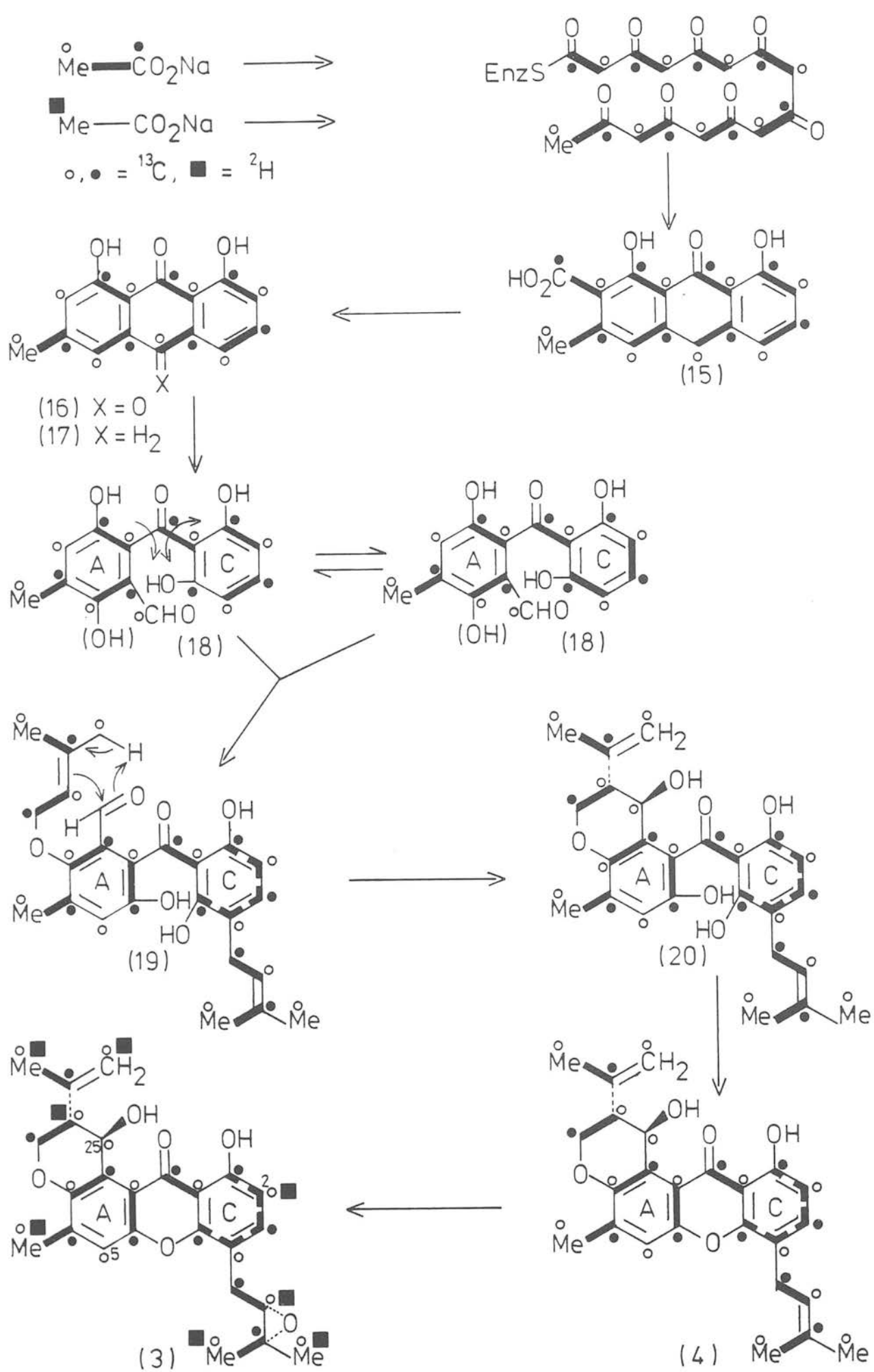
### 5.1 Background Information

The isolation of tajixanthone ( $C_{25}H_{26}O_6$ ) and shamixanthone ( $C_{25}H_{26}O_5$ ) along with several other xanthonenes from cultures of *Aspergillus variegator* was first reported<sup>1</sup> in 1970. Structures (1) and (2) respectively were proposed<sup>2</sup> for these two metabolites. However, a subsequent detailed examination of their spectral and chemical characteristics resulted in a fairly drastic revision<sup>3</sup> of the original structures to (3) and (4). This revision was based primarily on evidence from n.m.r. and infra-red studies of both the parent compounds and of derivatives and degradation products.

The similarities between these new structures and those of a group of metabolites first obtained<sup>4,5</sup> from *Aspergillus rugulosus*, the arugosins A, B and C; (5), (6) and (7), were apparent and the subsequent isolation<sup>6</sup> of arugosins A and B from *A. variegator* provided further circumstantial evidence that this group of metabolites was biogenetically related to tajixanthone and shamixanthone. The same strain of *A. variegator* also produced<sup>6</sup> three new compounds of related structure, the variecoxanthonenes A, B and C; (8), (9) and (10) respectively, and further work<sup>7</sup> on various other strains led to the characterisation of a further seven closely related minor metabolites.



It was suggested<sup>8</sup> that the benzophenone (11), present in its two hemiacetal forms as arugosins A and B, could be an intermediate in the biosynthesis of tajixanthone and shamixanthone. Cyclodehydration would provide the xanthone nucleus and the dihydropyran ring would be formed from the *ortho*-prenyloxy-aldehyde moiety. Interestingly, the benzaldehyde (12) obtained by the oxidation of variecoxanthone A could be isomerised by mild acid treatment to (+)-de<sup>S</sup><sub>7</sub>-C-prenylepishamixanthone<sup>6</sup> (13). The stereospecific nature of this conversion was rationalised by postulating a synchronous "ene-reaction", and consideration of two possible transition states led to the conclusion that the formation of the observed product, with *cis* disubstitution at C-20 and C-25 relative to the dihydropyran ring, was favoured due to a high energy electrostatic interaction between the xanthone and aldehyde carbonyl groups in the transition state required for the *trans* product. If this conclusion is valid, an explanation is required for the *trans* stereochemistry of shamixanthone itself. It was suggested<sup>6</sup> that hydrogen bonding of the carbonyls by the enzyme or enzymes controlling the reaction might alleviate the electrostatic interaction or, alternatively, the formation of the dihydropyran ring could occur before construction of the xanthone nucleus, allowing the benzophenone carbonyl to avoid the aldehyde function by rotation out of the plane of the aromatic ring. One strain of *A. variegata* yielded<sup>7</sup> small quantities of epi-isoshamixanthone (14) in which the substitution of the dihydropyran ring is *cis* and the prenyl group is at the C-2 position.



Scheme 1



The biosynthesis of the *A. variegata* xanthenes was first investigated<sup>8</sup> by the incorporation of [1-<sup>13</sup>C]- and [2-<sup>13</sup>C]acetates into tajixanthone. Rigorous assignment of the <sup>13</sup>C n.m.r. spectrum was first required and was accomplished primarily by comparison with the spectra of several derived compounds, by off-resonance proton-decoupling and by lanthanide-induced shifts. A labelling pattern was revealed (Scheme 1) that was consistent with the derivation of the xanthone nucleus from an octaketide anthrone precursor and with the mevalonate origin of two additional C<sub>5</sub> units.

These studies were followed by a report<sup>9</sup> of the incorporation of [1,2-<sup>13</sup>C<sub>2</sub>]- and [<sup>2</sup>H<sub>3</sub>]acetates into tajixanthone. The combined results of these biosynthetic experiments and the pathway that they suggest are shown in Scheme 1. The lack of deuterium label at C-25 of tajixanthone derived from [<sup>2</sup>H<sub>3</sub>]acetate implied that the necessary ring cleavage occurred on an anthraquinone intermediate such as (16) rather than on the corresponding chrysophanol anthrone (17) as originally proposed<sup>8</sup>. Deuterium was also missing at C-5, although present at C-2, suggesting that decarboxylation of the acyl terminal of the octaketide chain occurred after formation of an aromatic species such as (15). The two alternative carbon-labelling patterns revealed by the double-labelled experiment showed that ring C of tajixanthone (3) had been symmetrical and free to rotate around the bond to the

carbonyl group at some stage, indicating that the proposed benzophenone intermediate (18) did not yet possess the C-prenyl group.

Another group has studied<sup>10</sup> the incorporation of [1-<sup>13</sup>C]acetate into shamixanthone (4) produced by *A. rugulosus*. The results were consistent with the pathway described above, but were interpreted by the authors as evidence for a heptaketide origin for the xanthone nucleus.

The structure and biogenesis of a wider range of related fungal metabolites has been discussed in detail elsewhere<sup>11</sup>.

## 5.2 Origin of the Oxygen Atoms of Tajixanthone and Shamixanthone

### 5.2.1 Object of Experiment

The initial object of the present studies was to investigate the origin of the oxygen atoms of tajixanthone (3) and shamixanthone (4) using the recently introduced technique of <sup>18</sup>O-induced isotope shifts in the <sup>13</sup>C n.m.r. spectrum (Section 2.4.3.2). The method has already been applied to the xanthonenes ravenelin<sup>12</sup> and sterigmatocystin<sup>13,14</sup>. It was hoped that this would provide some evidence on the mechanisms of the processes depicted in Scheme 1. A previous attempt to incorporate [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate into tajixanthone had met with only limited success<sup>15</sup>. Owing to the low levels of incorporation achieved, an isotopically shifted signal was seen only for C-13.

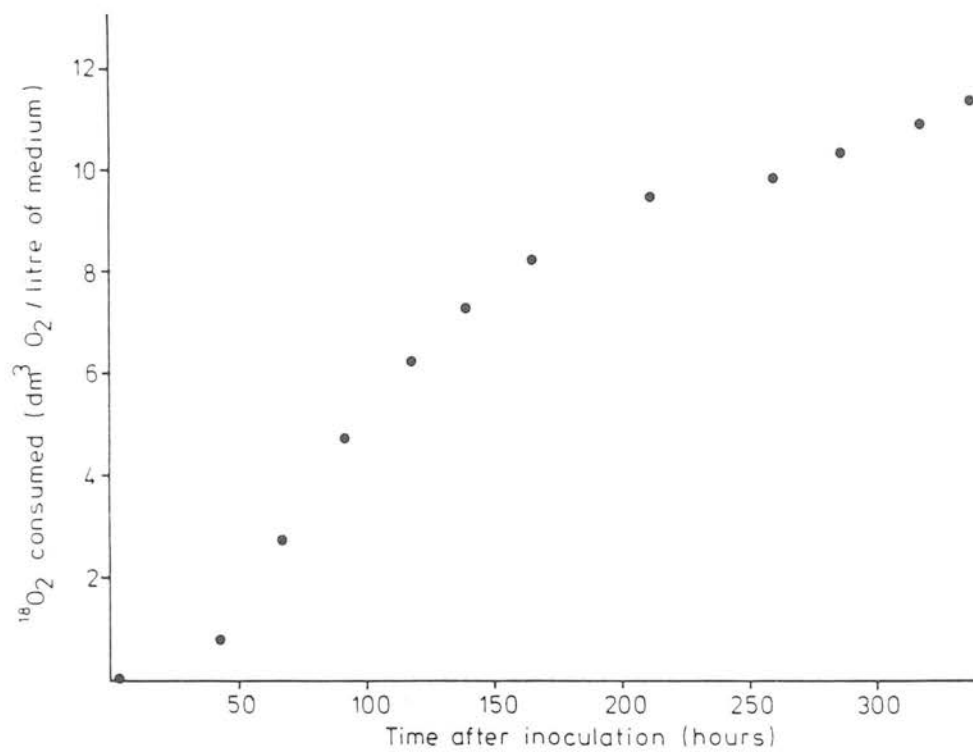


Figure 1      Uptake of  $\text{O}_2$  gas by cultures of *Aspergillus variegatus*  
212 K

### 5.2.2 Incorporation of $^{18}\text{O}_2$

It was decided to use  $^{18}\text{O}_2$  gas as a labelled precursor. Accordingly, *Aspergillus variegator* was grown as a surface culture using apparatus which allowed the circulation of a restricted atmosphere and measurement of the oxygen consumption of the fungus (Appendix). Preliminary experiments revealed that tajixanthone production was sensitive to periods of reduced oxygen partial pressure and the original apparatus was therefore modified to avoid these. The fungus consumed a surprisingly large volume of oxygen, particularly during the period of rapid mycelial growth (Figure 1).

In an attempt to economise on  $^{18}\text{O}_2$  gas, a fermentation was carried out in which the  $^{16}\text{O}_2$  of the initial atmosphere was only replaced by  $^{18}\text{O}_2$  after 164 hours, by which time replicatory growth of the mycelium had slowed. Mass spectrometry of the resulting tajixanthone, however, showed a negligible incorporation of label. The experiment was repeated on a smaller scale using  $^{18}\text{O}_2$  gas (of 98.7% purity) throughout the fermentation. Tajixanthone thus derived showed a major parent-ion peak at  $m/z = 430$ , 8 mass-units heavier than unenriched material, indicating the incorporation of four atoms of  $^{18}\text{O}$  per molecule. Shamixanthone, parent ion at  $m/z = 412$  ( $M + 6$ ), was similarly shown to contain three  $^{18}\text{O}$  atoms per molecule.

Table 1 Isotopically shifted  $^{13}\text{C}$  n.m.r. signals of tajixanthone and shamixanthone from a fermentation under  $^{18}\text{O}_2$

Carbon	Tajixanthone (3)			Shamixanthone (4)		
	$\delta^a$	$\Delta\delta^b$	$^{16}\text{O}_2: ^{18}\text{O}_2^c$	$\delta^a$	$\Delta\delta^b$	$^{16}\text{O}_2: ^{18}\text{O}_2^c$
1	160.4	1.0	79:21	159.7	1.0 <sup>d</sup>	
10	152.9	2.3	80:20	152.8	2.5	71:29
11	152.0	2.4	77:23	152.2	2.3	73:27
7	149.5	1.5	61:39	149.4	1.6	53:47
19	64.5	2.3	66:34	64.5	2.5	55:45
15	63.24	3.3	64:36			
25	63.16	1.5	66:34	63.2	1.5	60:40
16	58.5	4.1	64:36			

<sup>a</sup> In p.p.m. downfield from TMS in 90.6 MHz spectrum ( $\text{CDCl}_3$ )

<sup>b</sup> In p.p.m./100, upfield from unshifted signal

<sup>c</sup> Ratio of integrated intensities ( $\pm 5\%$ )

<sup>d</sup> Not resolved completely

To establish the positions of the  $^{18}\text{O}$  atoms in the labelled metabolites, analyses of the  $^{13}\text{C}$  n.m.r. spectra were required. Because the  $^{18}\text{O}$ -induced shifts are very small, ranging from zero to 0.05 p.p.m.<sup>16,17</sup>, they can only be detected in samples also containing unlabelled material as an internal reference; hence the spectra were obtained in each case from a mixture of labelled and unlabelled metabolite. Relevant sections of the 90.6 MHz  $^{13}\text{C}$  n.m.r. spectra are shown in expanded form (Figures 2 and 3) and the data are summarised in Table 1. The assignments are those previously published<sup>8</sup> except for C-1 and C-10, the assignments of which were reversed<sup>18</sup> on evidence from the incorporation of double  $^{13}\text{C}$ -labelled acetate<sup>9</sup>. The magnitudes of the observed  $^{18}\text{O}$  isotope shifts for these two carbons are consistent with the new assignments: C-1 would be expected to show the smaller shift by analogy with previous observations<sup>16,17</sup>. In the case of tajixanthone, there are two very close signals, at 63.24 and 63.16 p.p.m., previously<sup>8</sup> each assigned to one or other of C-15 and C-25. The size of the  $^{18}\text{O}$ -induced shifts suggests that the lower field signal be assigned to C-15. This signal shows an isotope shift comparable to the shift observed for C-16.

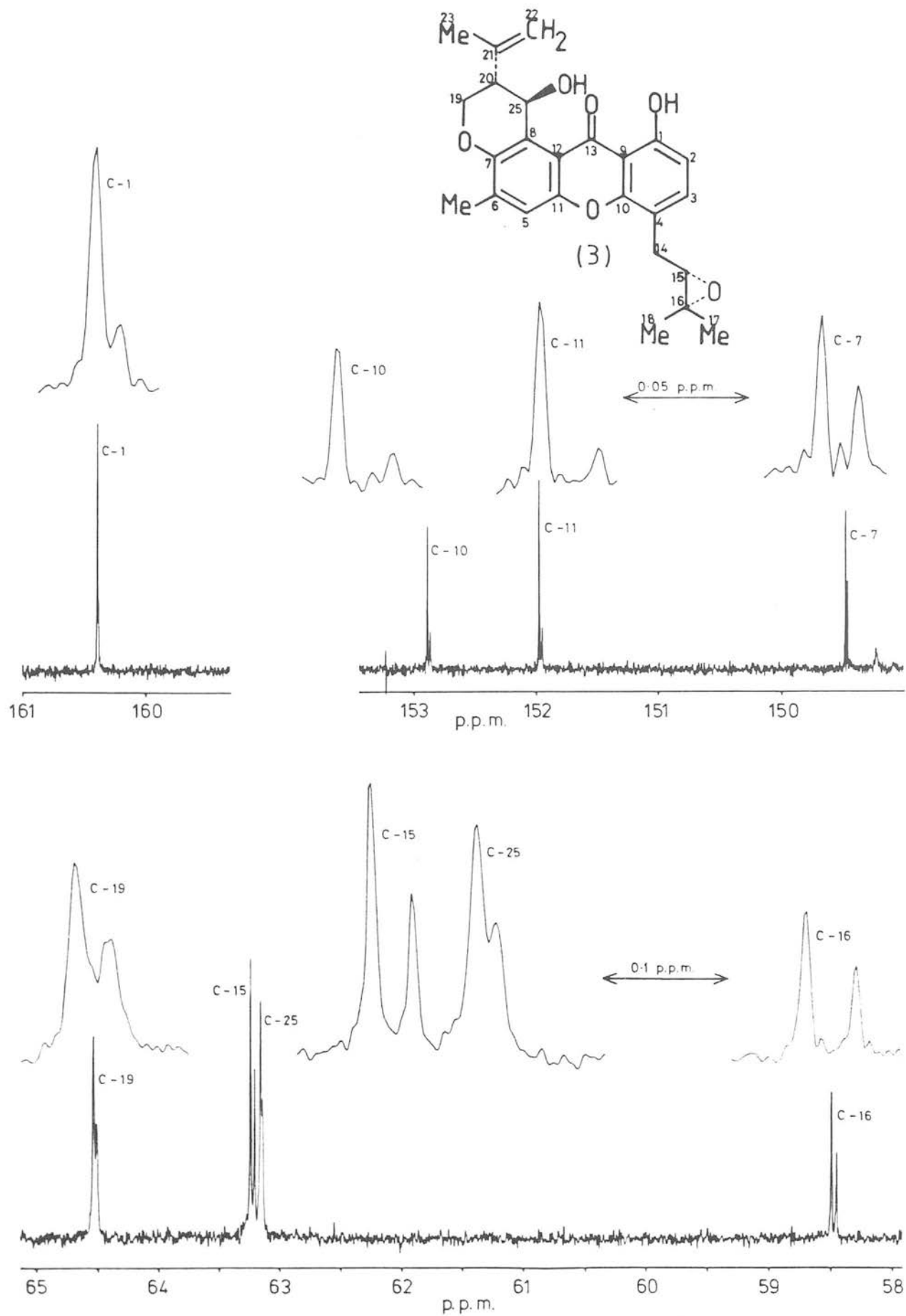


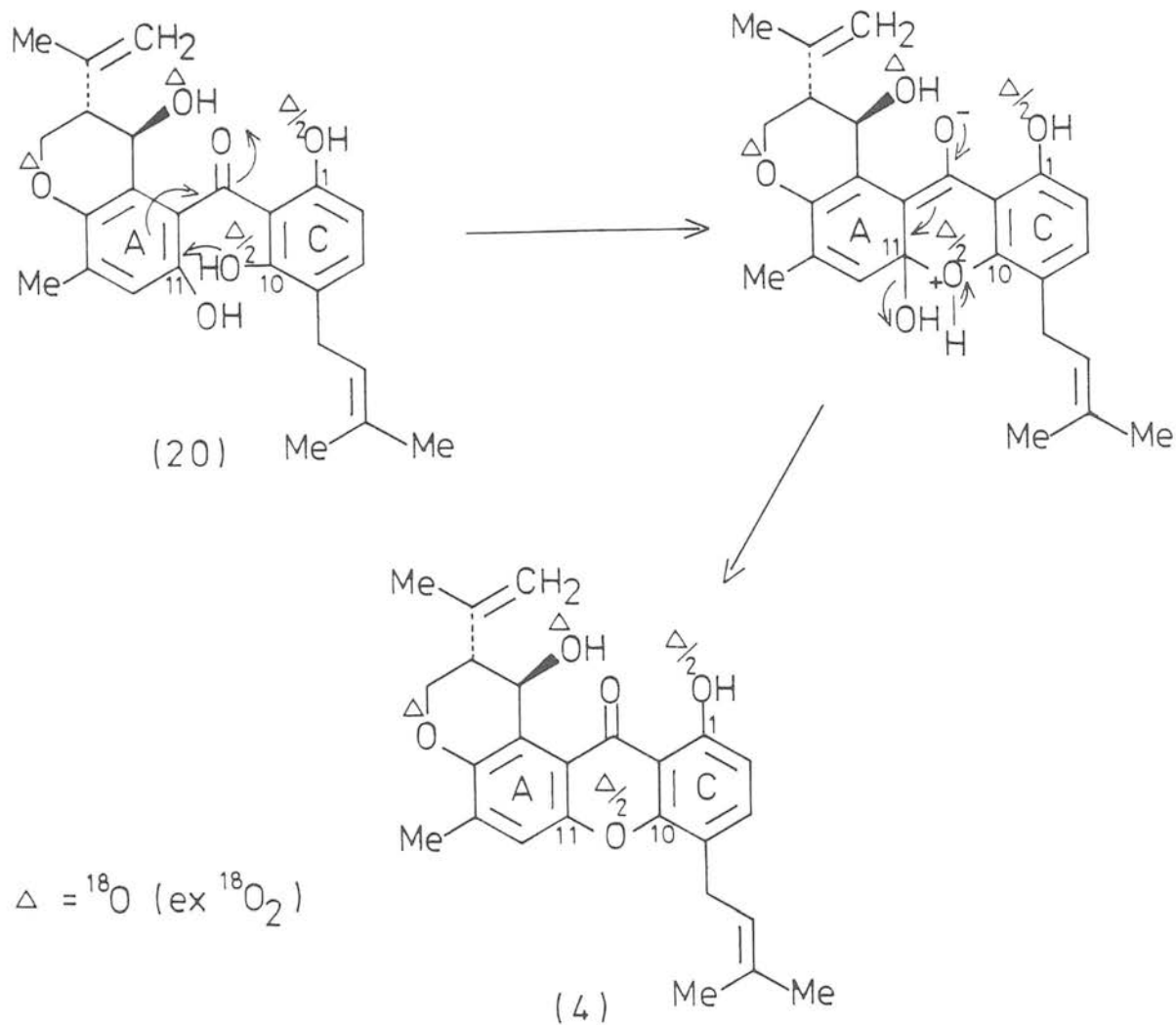
Figure 2 Sections of the 90.6 MHz <sup>13</sup>C n.m.r. spectrum of tajixanthone labelled by <sup>18</sup>O<sub>2</sub> gas

### 5.2.3 Results and Conclusions

With the exception of C-13, all the oxygen-bearing carbons of tajixanthone show (Figure 2) isotopically-shifted resonances, indicating the presence of atmospherically-derived oxygen at all of the oxygen functions other than the carbonyl group. However, the ratio of the intensities of the shifted and unshifted signals for C-1, C-10 and C-11 is, within experimental error, about half of that for the other label-bearing carbons. Thus, although a total of five oxygen sites are labelled in the sample as a whole, the relative amounts of labelling together with the mass spectral data show that in any one labelled molecule either the oxygen at C-1 or the oxygen between C-10 and C-11, but not both, is an  $^{18}\text{O}$  species. This supports the proposed pathway (Scheme 1), whereby the symmetrically substituted dihydroxyphenyl ring of the benzophenone precursor (18) is free to rotate before formation of the xanthone nucleus. One of the hydroxy groups of this ring is probably acetate derived; the other arises from atmospheric oxygen.

The equal amounts of  $^{18}\text{O}$ -labelling at C-1 and C-10 also indicate that on formation of the xanthone nucleus from a benzophenone, *e.g.* (19) or (20), it is the hydroxyl oxygen of ring C, rather than that of ring A which forms the ether linkage. The ring-A hydroxyl is expected to be acetate derived: should it form the ether linkage, no shifted signals would be anticipated for C-10 or C-11.





Scheme 2

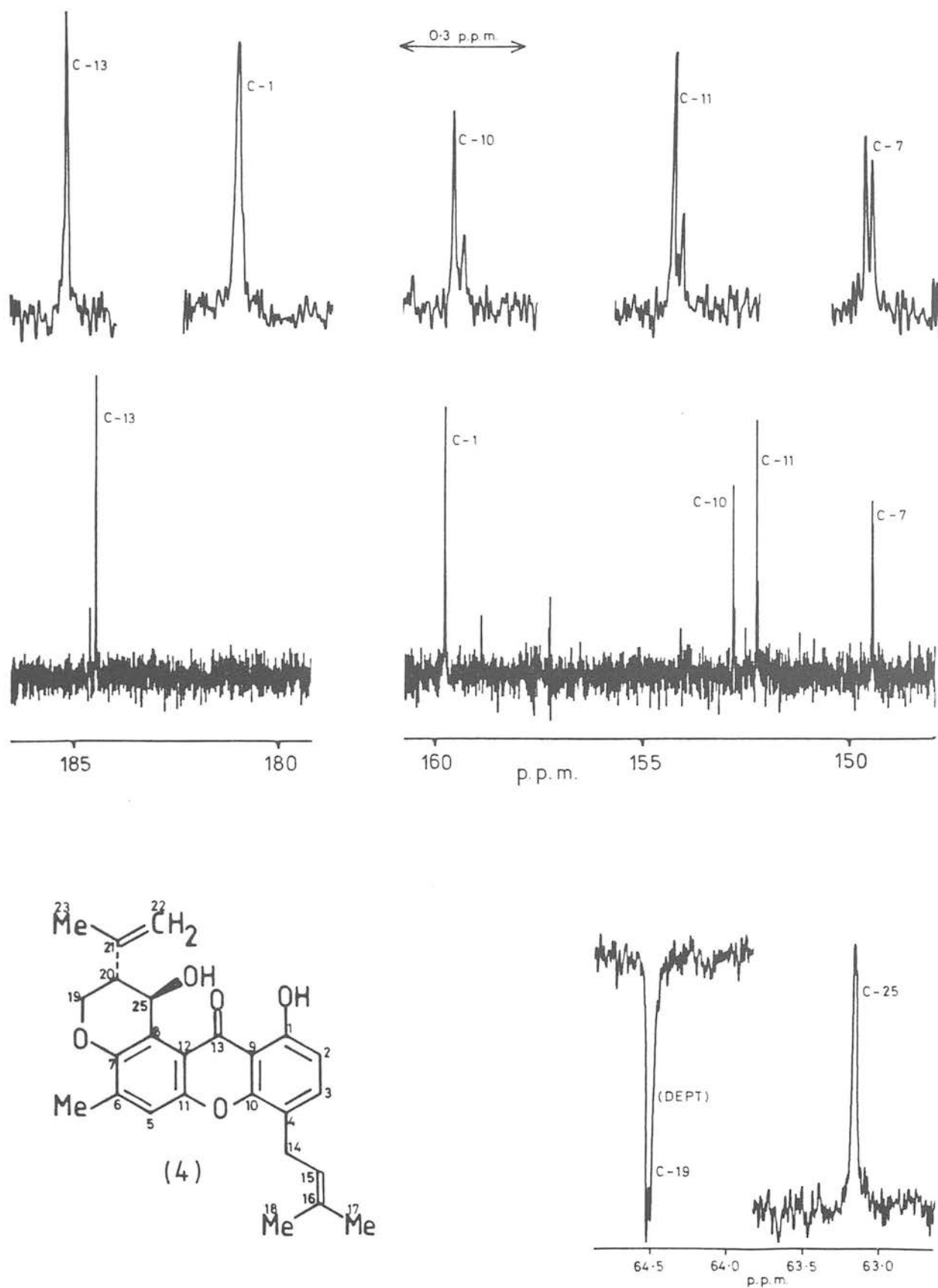
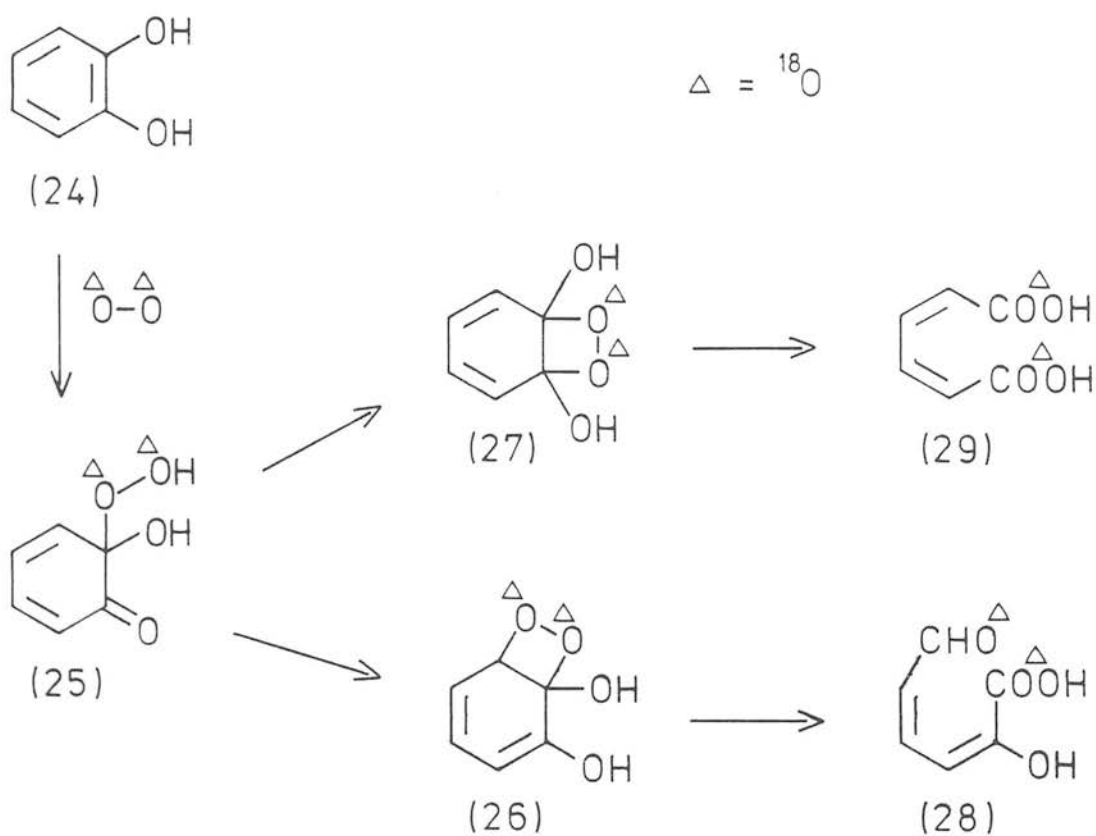
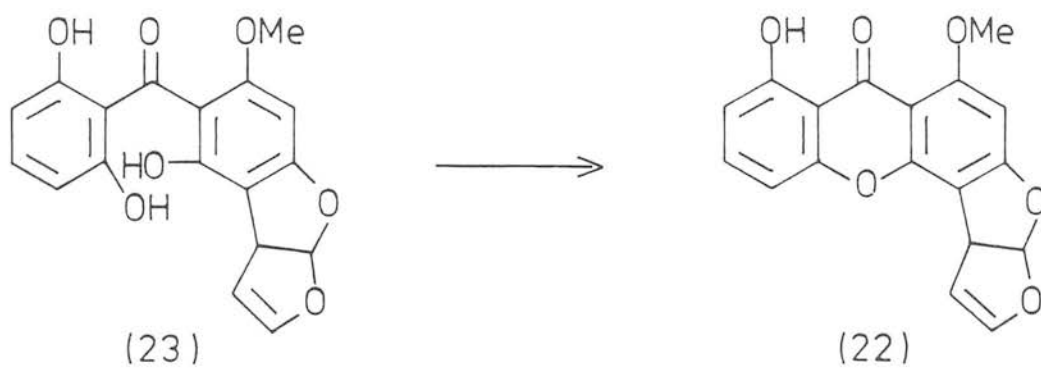
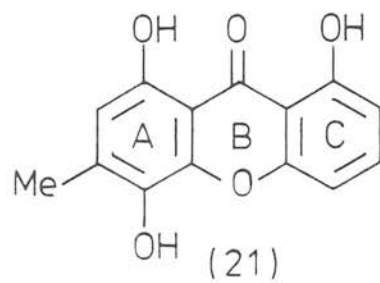


Figure 3 Sections of the 90.6 MHz  $^{13}\text{C}$  n.m.r. spectrum of shamixanthone labelled by  $^{18}\text{O}_2$  gas

A plausible mechanism for this ring closure is thus *via* a Michael addition-elimination process whereby the ring-C hydroxyl attacks the ring-A carbon, followed by loss of the ring-A hydroxyl group at C-11 (Scheme 2). Such reactions are known to occur readily<sup>19</sup>. Cyclisation in the reverse manner, whereby the ring-A hydroxyl displaces the one on ring C, cannot be entirely ruled out because of the experimental error in the intensity measurements but it can be no more than a minor route.

On the basis of these proposals,  $^{18}\text{O}$  isotope shifts for C-1 and C-10, although not C-11, would have been expected in the experiment<sup>15</sup> with  $[1\text{-}^{13}\text{C}, ^{18}\text{O}_2]\text{acetate}$ . That a shift was only seen for C-13 probably reflects the reduced intensity of the other signals (assuming roughly equal loss by exchange throughout the molecule) and their smaller shifts, especially in the case of C-1.

Examination of the  $^{13}\text{C}$  spectrum (Figure 3) of shamixanthone (4) labelled from  $^{18}\text{O}_2$  confirmed that the same situation prevailed. In this case it was not possible to fully resolve the isotopic shift for C-1, and so an accurate assessment of the  $^{16}\text{O}:^{18}\text{O}$  ratio was not feasible, but the abundance of  $^{18}\text{O}$  between C-10 and C-11 was again about half of that at the other labelled sites in the molecule. C-25, unambiguously assigned<sup>8</sup> for shamixanthone, shows the same size of isotope shift, 0.015 p.p.m., as does the tajixanthone signal at 63.16 p.p.m., providing further evidence that this signal should be assigned to C-25 of tajixanthone.



Scheme 3

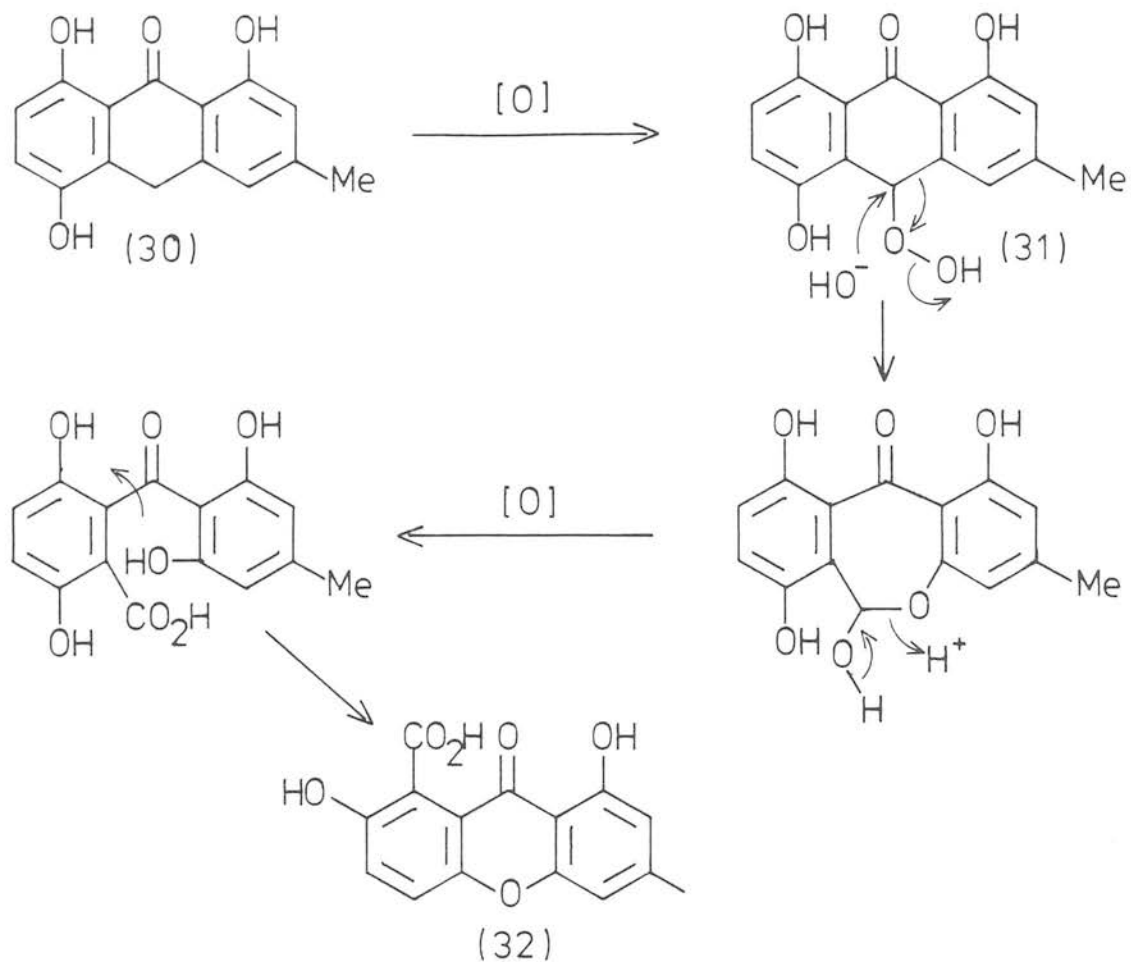
Overall, these results are consistent with similar observations of the related metabolite ravenelin<sup>12</sup> (21). Observations of <sup>18</sup>O isotope shifts of ravenelin derived from [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate showed that the xanthone ring formation occurred with retention of the acetate-derived <sup>18</sup>O label of the ring-C hydroxyl. Ravenelin also shows scrambling of the carbon-labelling of this ring<sup>20</sup> and must be considered a good biosynthetic parallel to tajixanthone and shamixanthone.

The same Michael addition-elimination mechanism was proposed<sup>13</sup> for the formation of the xanthone ring of sterigmatocystin (22) from a suggested benzophenone intermediate (23) containing a symmetrically substituted ring, but, as no scrambling of <sup>13</sup>C label is observed in this ring, other mechanisms avoiding such symmetry have since been suggested<sup>14,21</sup>.

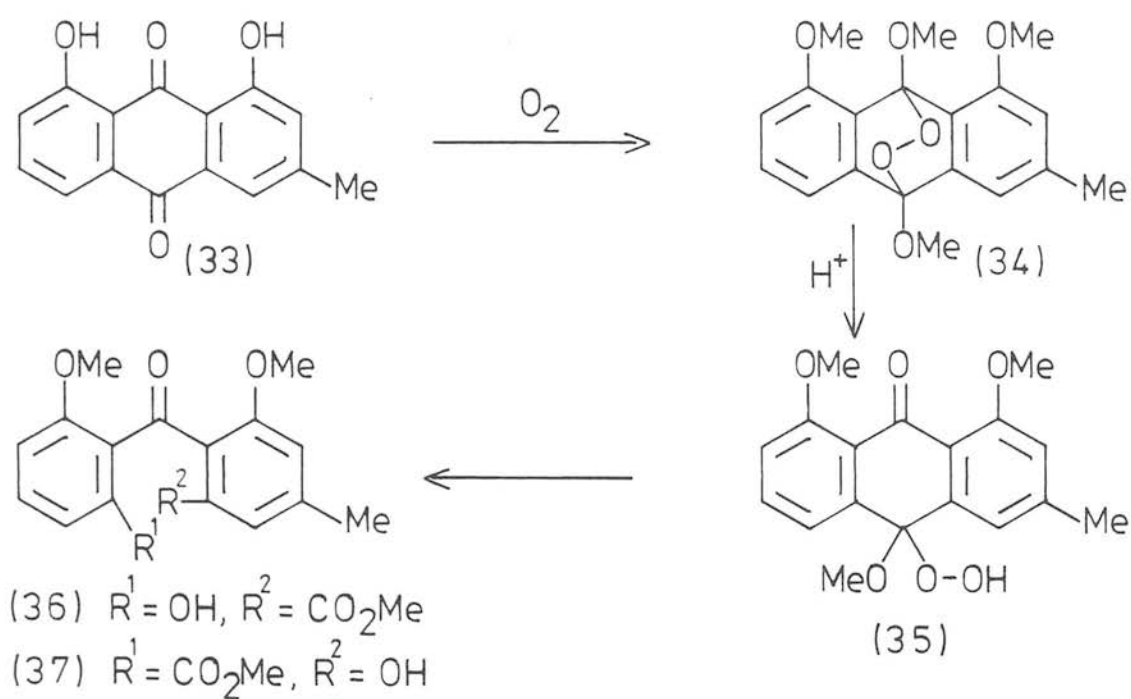
### 5.3 Mechanism of the Ring-Cleavage

#### 5.3.1 Background to Experiment

Anthrone and anthraquinone intermediates are widely<sup>12</sup>, although not exclusively<sup>22</sup>, implicated in the biogenesis of fungal xanthenes. However, the required fission of the central ring is poorly understood. Biomimetic studies have been widely employed in the general investigation of the ring cleavage of aromatic compounds and this work has been comprehensively reviewed<sup>23</sup>. Enzymic oxidations of catechol (24) to muconic acid (29) and an isomeric



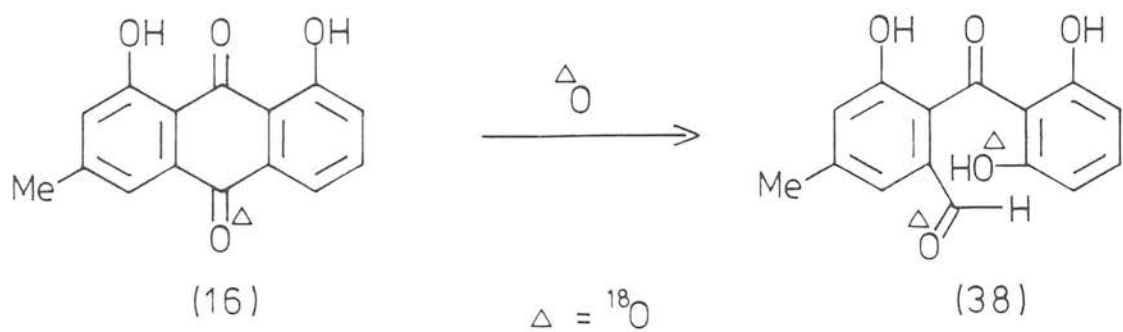
Scheme 4



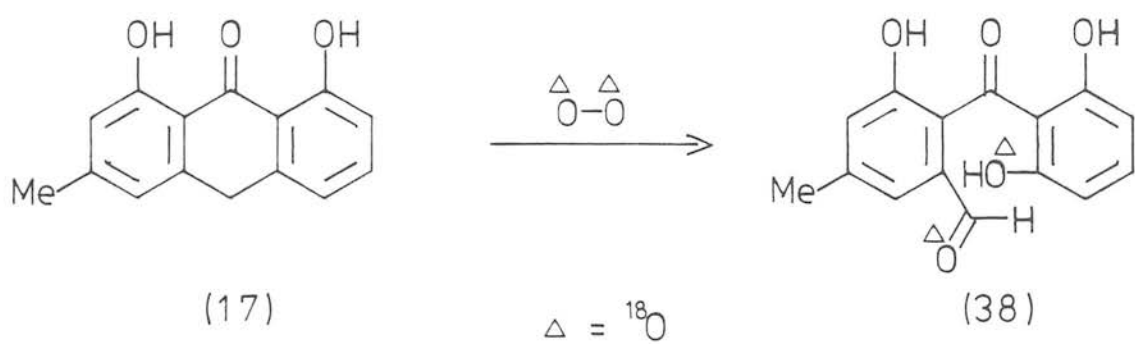
Scheme 5

aldehyde (28) are known<sup>23</sup> to result in the incorporation of the two atoms of a labelled oxygen molecule into the carbonyl groups of the products, and this has been rationalised by invoking hydroperoxide (25) and/or dioxetane, (26) and (27), intermediates produced by the action of a dioxygenase enzyme (Scheme 3). Following the early suggestion<sup>24</sup> that a hydroperoxide (31) could be implicated in a proposed biosynthesis of the xanthone pinselic acid (32) from helminthosporin anthrone (30) (Scheme 4), biomimetic experiments on the ergochromes<sup>25,26</sup> resulted in the preparation of an endoperoxide (34) from an anthraquinone (33) using singlet oxygen. An acid catalysed rearrangement subsequently gave the secoanthraquinones, (36) and (37), *via* the hydroperoxide (35) (Scheme 5).

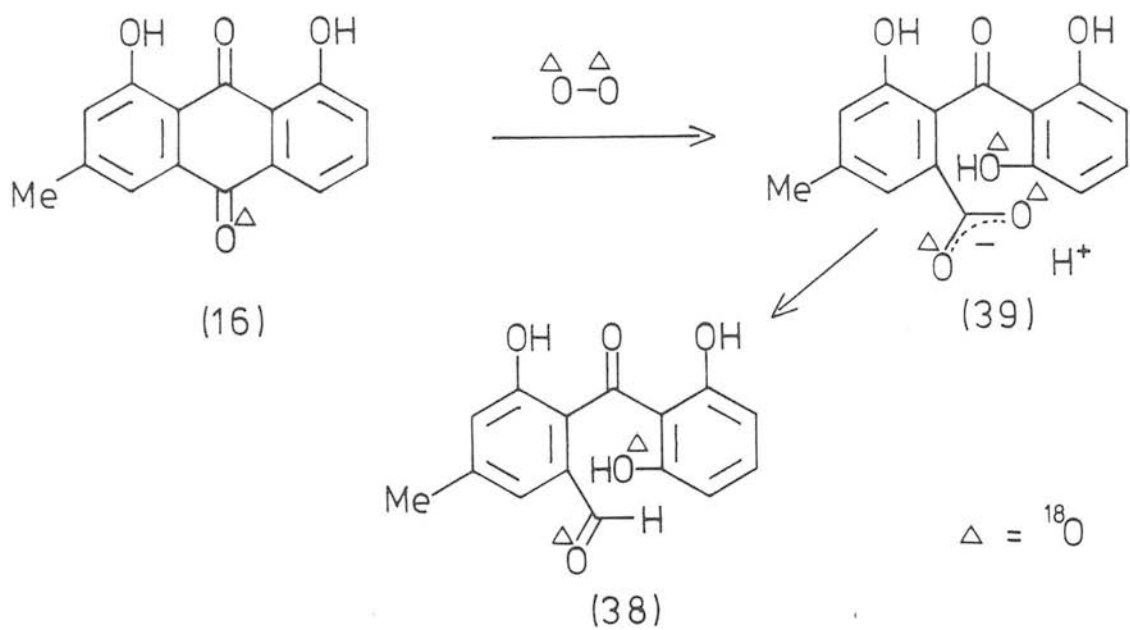
Ergochrome biosynthesis<sup>26</sup> would appear to be closely related to that of tajixanthone (3) and shamixanthone (4). As both of the oxygen atoms introduced in the course of the cleavage of the anthraquinone (16) or anthrone (17) precursor are, according to the experiment earlier described, wholly derived from atmospheric oxygen, a mechanism as in Scheme 4 where an anthrone hydroperoxide (31) undergoes nucleophilic attack by hydroxide ion is ruled out. Thus, if such an endoperoxide or dioxetane type mechanism is operative in tajixanthone biosynthesis, both of the oxygen atoms finally incorporated might come from the same molecule of atmospheric oxygen. It is possible to devise an experiment, using a judicious mix of  $^{16}\text{O}_2$  and  $^{18}\text{O}_2$ , which can check whether this is the case.



Scheme 6



Scheme 7



Scheme 8



Brefeldin A<sup>27</sup>, cochlioquinones A and B<sup>28</sup>, ipomeamarone<sup>29</sup> and prostaglandin E<sub>1</sub><sup>30</sup> have been investigated in this way, and, in the last example, a dioxygenase was implicated as a result.

### 5.3.2 Design of $^{16}\text{O}_2$ - $^{18}\text{O}_2$ Mixed Atmosphere Experiment

Each molecule of tajixanthone (3) contains four atmospherically-derived oxygen atoms. The distribution of parent-ion masses among the five possibilities of M to M + 8 if all four oxygens are independently introduced is given by the binomial expansion of  $(a + b)^4$ , where "a" is the  $^{16}\text{O}_2$  mole fraction of the total  $\text{O}_2$  present and "b" is the corresponding  $^{18}\text{O}_2$  fraction. Such a distribution would pertain to a ring cleavage mechanism whereby an anthraquinone species (16) was subject to mono-oxygenation (Scheme 6). If, however, two of the oxygen atoms were introduced together *via* (say) a dioxygenase acting on an anthrone (17) (Scheme 7), the ring cleavage oxygenation would have probability = b of introducing two  $^{18}\text{O}$  atoms and probability = a of introducing none. The other two relevant oxygenations would give zero, one or two labels according to the expansion of  $(a + b)^2$ .

A third possibility, illustrated in Scheme 8, is that of a dioxygenase process acting on an anthraquinone (16) and resulting in the benzoic acid (39). Subsequent

Table 2 Distributions of labelling probabilities for  
tajixanthone derived by three hypothetical ring-  
cleavage mechanisms

Parent Ion	Scheme 6	Probability Scheme 7	Scheme 8
M	$a^4$	$a^3$	$\frac{1}{2}(a^4 + b^3)$
M + 2	$4a^3b$	$2a^2b$	$\frac{1}{2}(4a^3b + 2a^2b)$
M + 4	$6a^2b^2$	$ab^2 + ba^2$	$\frac{1}{2}(6a^2b^2 + ab^2 + ba^2)$
M + 6	$4ab^3$	$2b^2a$	$\frac{1}{2}(4ab^3 + 2b^2a)$
M + 8	$b^4$	$b^3$	$\frac{1}{2}(b^4 + b^3)$

$a = {}^{16}\text{O}_2$  mole fraction of total  $\text{O}_2$

$b = {}^{18}\text{O}_2$  mole fraction of total  $\text{O}_2$

reduction to the benzaldehyde (38) would require the loss of either (i) one of the pair of newly introduced oxygens with results equivalent to those of Scheme 6, or (ii) the non-ketide quinone oxygen of (16), equivalent to Scheme 7. Hence, assuming an atmospheric origin for this latter oxygen atom and equal likelihood of either process (i) or (ii), the distribution of molecules of mass M to M + 8 resulting from Scheme 8 is simply the average of the distributions according to Schemes 6 and 7.

Assuming also that exchange of  $^{18}\text{O}$  from the carbonyl groups of (38) or (39) is negligible in each case and ignoring the effects of  $^{13}\text{C}$ , the expected distributions are given by Table 2.

Because of the natural abundance of  $^{13}\text{C}$  (1.1%) only 75.8% of the intensity of a  $\text{C}_{25}$  parent-ion peak calculated as above actually appears at its "original" mass. The rest is distributed to higher masses and 2.8% is transferred to the peak two mass-units greater. (Given by the binomial expansion:

$$(0.989 + 0.011)^{25} = 0.758 + 0.211 + 0.028 + 0.0024 + 0.00015 \text{ etc.)}$$

Thus the intensity actually expected for any  $\text{C}_{25}$  peak would be 75.8% of that derived from Table 2 plus 2.8% of the intensity similarly derived for the corresponding peak (if any) two mass-units lower. Preliminary calculations on this basis revealed that production of tajixanthone under an

atmosphere of 74.8%  $^{16}\text{O}_2$  and 25.2%  $^{18}\text{O}_2$  would result in a good differentiation between the three cases considered, giving the intensity of the peak at M equal to that at M + 2 for Scheme 8, less than that at M + 2 for Scheme 6 and greater than that at M + 2 for Scheme 7.

In the actual experiment, 25% of the oxygen used was from the  $^{18}\text{O}_2$  cylinder (98.7% isotopic purity) and 75% was  $^{16}\text{O}_2$ , giving a mix of 24.7%  $^{18}\text{O}_2$  and 75.3%  $^{16}\text{O}_2$  (assuming all the impurity in the  $^{18}\text{O}_2$  was  $^{16}\text{O}_2$ ). The fungus was grown entirely under an atmosphere containing this mix of oxygen. Using these figures, results were predicted for each mechanistic possibility considered above and for both tajixanthone and shamixanthone (Table 3). The calculation for shamixanthone was slightly simpler because only three oxygens were considered but was otherwise identical.

It is rather more likely that the isotopic impurity in the  $^{18}\text{O}_2$  cylinder consisted of molecules containing both an  $^{18}\text{O}$  and a  $^{16}\text{O}$  atom. These could be present up to a maximum extent of 2.6% of all the  $\text{O}_2$  species present in the cylinder. In this case the growth atmosphere would have comprised  $^{18}\text{O}_2$ ,  $^{16,18}\text{O}_2$  and  $^{16}\text{O}_2$  in the ratio of 24.35:0.65:75.00. Labelling by mono-oxygenation processes would be unaffected by this but a dioxygenation process could now result in the incorporation of two, one or zero  $^{18}\text{O}$  atoms with probabilities in the same ratio.

Table 3 Predicted and observed relative intensities of parent-ion peaks

Tajixanthone parent ion	Relative Intensities <sup>a</sup>			
	Scheme 6	Scheme 7 <sup>b</sup>	Scheme 8 <sup>b</sup>	Observed <sup>c</sup>
M	31.1	41.9 (41.8)	36.4 (36.4)	34.0 (±0.7)
M + 2	42.1	29.1 (29.3)	35.6 (35.8)	39.5 (±0.8)
M + 4	21.6	19.3 (19.3)	20.5 (20.5)	20.5 (±0.7)
M + 6	5.1	9.7 (9.6)	7.5 (7.3)	6.1 (±0.4)
M + 8	-	-	-	-

Shamixanthone parent ion	Relative Intensities <sup>a</sup>			
	Scheme 6	Scheme 7 <sup>b</sup>	Scheme 8 <sup>b</sup>	Observed <sup>c</sup>
M	42.1	58.7 (58.3)	50.1 (50.0)	42.4 (±2.2)
M + 2	42.9	21.4 (21.8)	32.4 (32.6)	39.4 (±2.1)
M + 4	15.1	19.9 (19.9)	17.4 (17.4)	18.2 (±1.8)
M + 6	-	-	-	-

<sup>a</sup> Even-mass ion intensities normalised to a total of 100 and ignoring in each case the highest-mass ion intensity as experimentally this was too small to measure.

<sup>b</sup> Figures in brackets show predicted results if 2.6% of the cylinder gas is <sup>16,18</sup>O<sub>2</sub> (see text)

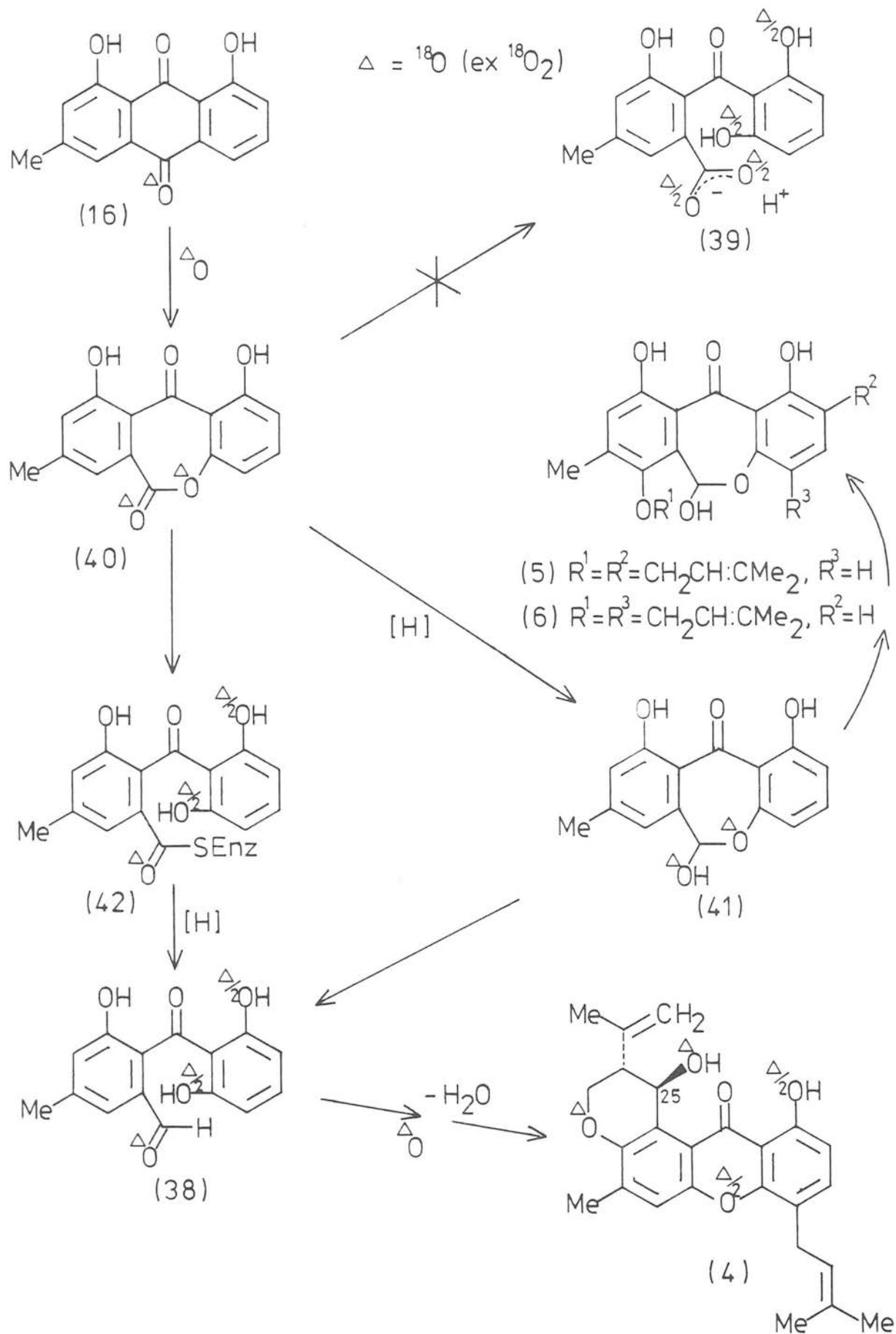
<sup>c</sup> Errors are based on the maximum possible effect of a 0.5 mm measuring error in the height of every parent-ion line.

Repeating the calculations for Schemes 7 and 8 on this basis gave the figures (Table 3) shown in brackets: it is apparent that any discrepancy arising from the presence of a small proportion of  $^{16,18}\text{O}_2$  species is very minor.

### 5.3.3 Results and Conclusions

The observed distributions of parent-ion masses from this experiment are also shown in Table 3. It is clear that they most closely match the figures expected from a pathway involving four independent mono-oxygenations as in Scheme 6. The match is not perfect and the discrepancy is larger than the estimated error. (This is based on the precision of measuring the peak heights.) However, the theoretical figures actually represent upper limits of labelling since they take no account of any possible exchange processes which would bias the distribution towards lower masses. As the experimental figures indicate labelling to an extent greater than the upper limits for the other mechanistic possibilities, these possibilities must be discounted.

These results thus appear to rule out a dioxygenase mechanism for the ring-cleavage where both oxygen atoms are subsequently retained. In addition, the results of the n.m.r. experiment (Section 5.2) indicate that no source other than the atmosphere provides a significant part of the oxygen which, in the final metabolites, is carried by the carbon atoms that formed the cleaved bond. At first sight

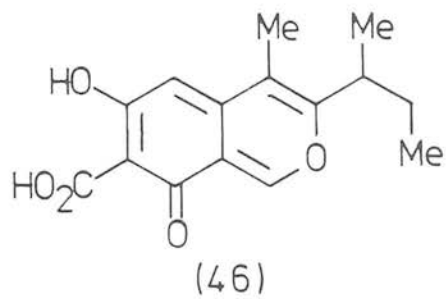
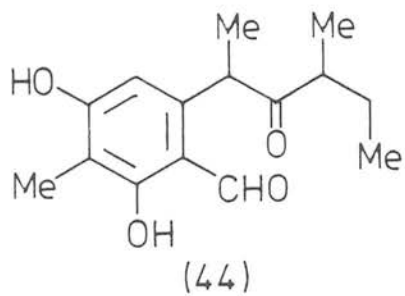
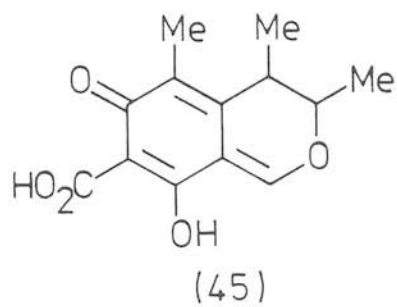
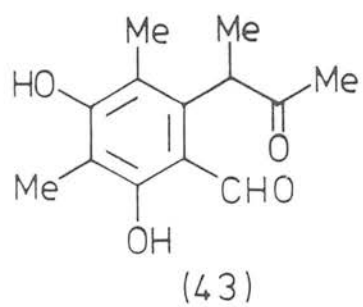


Scheme 9

it seems rather surprising, although fortunate from the standpoint of the mass spectrometry experiment, that negligible loss occurs of the oxygen label eventually found at C-25 of tajixanthone (3) and shamixanthone (4). This, and the other observations, could, however, be explained by a biological Baeyer-Villiger type oxidation<sup>31</sup> operating on an anthraquinone (16), itself derived by aerobic oxidation of the corresponding anthrone (17). The resulting lactone (40) would be relatively immune to exchange and, as a possible intermediate, has the additional attraction that direct reduction would give the hemiacetal structure (41) found in arugosins A (5) and B (6). Direct formation of the putative aldehyde intermediate (38) could follow, avoiding the loss of label inevitable if hydrolysis of the lactone (40) to the benzoic acid (39) was an intermediate step (Scheme 9). Benzaldehydes are also less susceptible to hydration than other aldehydes; hence formation of the dihydropyran ring could proceed without significant loss of  $^{18}\text{O}$  label at C-25.

Also shown in Scheme 9 is an alternative viable pathway whereby the lactone (40) gives the thioester (42) by transesterification. Reductive cleavage could then give the aldehyde (38). Again, simple hydrolysis of the lactone or of the thioester is precluded by the retention of  $^{18}\text{O}$  label at C-25. The direct formation of an aldehyde species by reductive cleavage of a corresponding thioester,





rather than by a hydrolysis-reduction sequence has been previously suggested. On the basis of incorporation experiments with possible advanced intermediates, the aldehydes (43) and (44) were implicated as the first enzyme-free species in the biosyntheses of citrinin<sup>32</sup> (45) and ascochitine<sup>33</sup> (46).

#### 5.4 Experimental

(General procedures and instrumentation are described in Section 3.6.1).

*Production and isolation of tajixanthone and shamixanthone*

*Aspergillus variegator* - strain 212 K (derived from NRRL 212) was stored in the dark at 4°C under liquid paraffin on slopes of Czapek-Dox agar (Oxoid CM 95 plus 2% w/w Oxoid Agar No. 3, L 13). Cultures for large scale inoculations were prepared from these by growth, also on Czapek-Dox agar, in medical flats at 26°C in constant light for 8-10 days. A spore suspension in distilled water was subsequently used to inoculate the production medium of malt-extract broth (Oxoid Malt Extract, 3% w/v; Oxoid Mycological Peptone, 0.5% w/v; distilled water, to 100%), 100 ml of which was contained in each of the 500 ml Erlenmeyer flasks employed. The fungus was allowed to grow for 12-14 days, in constant light, at 26°C. As growth proceeded the initially white mycelial mat developed a dark green pigmentation.

The growth medium was separated from the mycelial mat by either decanting or filtration (Whatman's No. 1) and the mycelium was washed with a little water. After drying the mycelium overnight at 50°C, it was ground to a powder and extracted for several hours with distilled petroleum ether (b.p. 40-60°C) using a Soxhlet apparatus. This gave either a yellow or orange extract, the methanol soluble

constituents of which were separated by preparative t.l.c., developing with diethyl ether-benzene (5:95). One pass was usually sufficient: the yellow bands at  $R_f = 0.25$  (tajixanthone) and  $R_f = 0.50$  (shamixanthone) were each eluted with ethyl acetate to give in each case a crystalline yellow solid, typically  $180 \text{ mg l}^{-1}$  and  $50 \text{ mg l}^{-1}$  in respective yield, on evaporation of the solvent.

The tajixanthone sample required further purification by preparative t.l.c. (developing with methylene chloride) to remove an unidentified material which could not be visualized by either long (350 nm) or short (254 nm) wavelength u.v. light. Spraying an analytical plate with sulphuric acid-ethanol (20:80) and then warming it, produced a blue spot ( $R_f = 0.21$ ) above the yellow spot of tajixanthone ( $R_f = 0.06$ ). The purified tajixanthone (yield about  $100 \text{ mg l}^{-1}$ ) was recrystallised from petroleum ether (b.p.  $30-40^\circ\text{C}$ ) to give yellow needles, m.p.  $158.5 - 160.5^\circ\text{C}$  (lit.<sup>3</sup>,  $158 - 159^\circ\text{C}$ ). Shamixanthone was recrystallised from methanol, also to give yellow needles, m.p.  $151 - 153^\circ\text{C}$  (lit.<sup>3</sup>,  $154 - 156^\circ\text{C}$ ). In each case, spectral and physical data were in agreement with published values<sup>3,8</sup> and with those of authentic samples.

*Incorporation of  $^{18}\text{O}_2$  into tajixanthone and  
shamixanthone*

*Aspergillus variegator* - strain 212 K - was grown as previously described in 6 Erlenmeyer flasks. As an earlier experiment had indicated that use of the "closed volume" apparatus (for measuring  $\text{O}_2$  uptake - Appendix) with this culture resulted in reduced yields of tajixanthone ( $60 \text{ mg l}^{-1}$  compared with  $102 \text{ mg l}^{-1}$  from control culture), the "constant pressure" apparatus (Appendix) was used and 3 of the flasks were attached thereto. The other three were retained as controls. The experimental culture was grown under an atmosphere of  $^{18}\text{O}_2$  -  $^{14}\text{N}_2$  (20:80) consuming 3.40 litres of oxygen. After 337 hours tajixanthone and shamixanthone were isolated.

*Results*

	Control	Experimental
Weight of mycelium ( $\text{g l}^{-1}$ )	13.4	14.2
Weight of extract ( $\text{g l}^{-1}$ )	0.55	0.58
Weight of tajixanthone ( $\text{mg l}^{-1}$ )	107	107
Weight of shamixanthone ( $\text{mg l}^{-1}$ )	47	57

See Figure 1 (Section 5.2.2) for a plot of  $^{18}\text{O}_2$  consumption versus time.

In a previous experiment, the culture had started growth under  $^{16}\text{O}_2$  -  $^{14}\text{N}_2$  (20:80) and the  $^{16}\text{O}_2$  in the gas burette had only been replaced by  $^{18}\text{O}_2$  when the first trace of yellow pigmentation was discerned (164 hours).

After growth for a further 127 hours, during which time the fungus consumed 1.48 litres of oxygen (out of a total of 6.44 litres), the metabolites were isolated. However, mass spectrometry indicated negligible incorporation of  $^{18}\text{O}_2$ .

$^{18}\text{O}_2$  -  $^{16}\text{O}_2$  mixed atmosphere experiment

One flask only of *Aspergillus variegator* - strain 212 K - was grown as above under an atmosphere of  $\text{O}_2$  -  $\text{N}_2$  (26:74). Of the oxygen present, 25.0% was from an  $^{18}\text{O}_2$  cylinder (98.7% isotopic purity) and 75.0% was from a  $^{16}\text{O}_2$  cylinder. The two isotopic species were pre-mixed in the gas burette to ensure a constant isotopic ratio throughout growth.

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Appendix

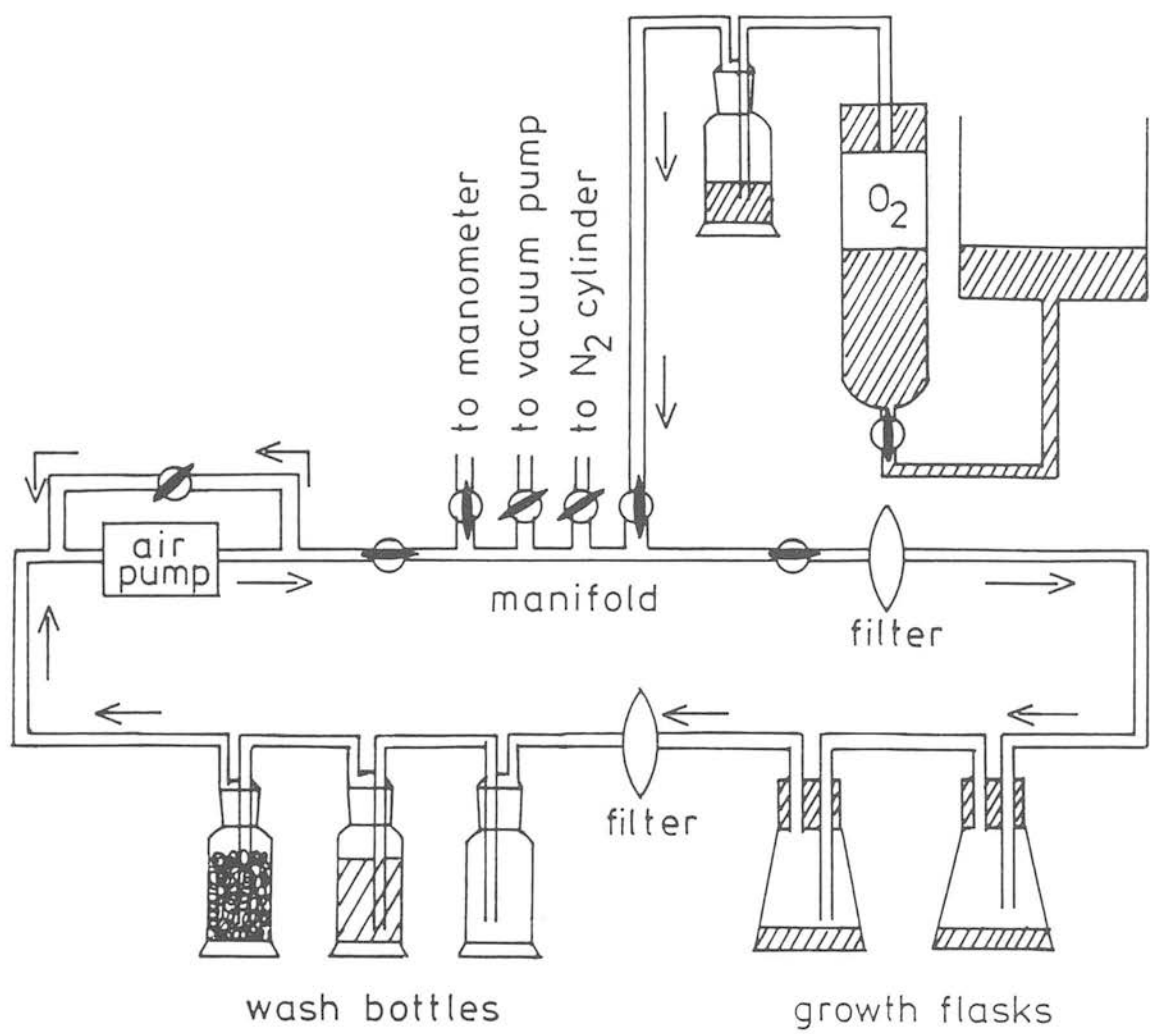


Figure 1 Oxygen-handling apparatus.

Apparatus for the Measured Utilisation  
of Molecular Oxygen by Fungal Cultures

The basic apparatus (see Figure 1) comprised an aquarium air-pump, (Rena 101) sealed into a polyester resin block with pipes for incoming and outgoing gases. The outgoing pipe was attached to a glass manifold which provided attachment for a manometer and incorporated stopcocks for gas sources and for a vacuum-pump. From the manifold the recirculating atmosphere passed successively through a bacterial filter, the growth flasks, another filter and a series of three Dreschel wash-bottles. The second of these contained 5M aqueous potassium hydroxide (130 ml) which absorbed the carbon dioxide produced by the culture. The first was empty and was connected back to front: in the event of the potassium hydroxide solution being accidentally siphoned back, the first wash-bottle acted as a trap and allowed the return of the solution to its correct bottle simply by turning on the air-pump. The last wash-bottle contained cotton wool and was intended as a filter to prevent any alkaline spray reaching the pump. The rate of flow (about  $50 \text{ ml min}^{-1}$ ) through the system was regulated by a hose clip on a length of rubber tubing connected in parallel across the air-pump.

In use, the consumption of oxygen was measured as a pressure drop by the manometer and the oxygen was replenished at intervals. This apparatus was used for some incorporation experiments and elsewhere in the text is referred to as the "closed volume" apparatus.

However, during periods of vigorous growth, this protocol resulted in nearly all the available oxygen being consumed over a short time, resulting on occasions in retarded growth and lowered yields of metabolites. Consequently the apparatus was modified for later experiments by the addition of a reservoir of oxygen contained in a large glass gas-burette and connected to the manifold *via* a Dreschel bottle partly filled with water. This acted as a one-way valve, allowing oxygen from the reservoir to flow into the circulating system as that oxygen already therein was consumed. Utilisation of oxygen was measured by noting the change in the level of the water in the gas-burette. Using this system, the pressure drop over the periods during which the experiment was unattended was negligible and growth was uninhibited. This is elsewhere referred to as the "constant pressure" apparatus.

At the start of an experiment, the assembled apparatus, including the growth flasks, was flushed through with nitrogen gas for a prolonged period to remove all oxygen. The system was then closed and a proportion (usually 20%) of the nitrogen atmosphere, as indicated by the manometer, was removed by a vacuum pump and replaced by either  $^{16}\text{O}_2$  or  $^{18}\text{O}_2$ . (By noting the volume of oxygen this required and knowing the pressure drop to which it corresponded, the total volume of the circulating system could be estimated.) The air-pump was then started and the experiment proceeded.

Publications

## **Structural Revision and Synthesis of LL-D253 $\alpha$ : a Chromanone Metabolite of *Phoma pigmentivora***

**C. Rupert McIntyre and Thomas J. Simpson\***

*Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland, U.K.*

LL-D253 $\alpha$ , a chromanone metabolite of *Phoma pigmentivora*, has been shown by analysis of the  $^1\text{H}$ -coupled  $^{13}\text{C}$  n.m.r. spectrum and by synthesis to be 7-hydroxy-8-(2-hydroxyethyl)-5-methoxy-2-methylchromanone.

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*Chemical Communications 1984*

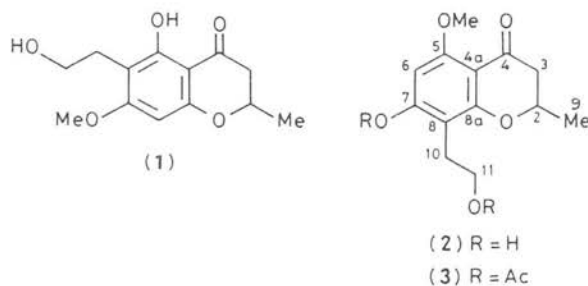
## Structural Revision and Synthesis of LL-D253 $\alpha$ : a Chromanone Metabolite of *Phoma pigmentivora*

C. Rupert McIntyre and Thomas J. Simpson\*

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LL-D253 $\alpha$ , a chromanone metabolite of *Phoma pigmentivora*, has been shown by analysis of the  $^1\text{H}$ -coupled  $^{13}\text{C}$  n.m.r. spectrum and by synthesis to be 7-hydroxy-8-(2-hydroxyethyl)-5-methoxy-2-methylchromanone.

LL-D253 $\alpha$  was first isolated from *Phoma pigmentivora* and was assigned the chromanone structure (1) on the basis of spectroscopic and degradative studies.<sup>1</sup> It has also been isolated along with a number of related co-metabolites from *Phoma violacea* and *Sclerotinia fructigena*.<sup>2</sup> We now report  $^{13}\text{C}$  n.m.r. studies on LL-D253 $\alpha$ , carried out as a preliminary to biosynthetic labelling experiments,<sup>3</sup> which necessitate revision of the structure to (2) on the basis of a full analysis of the fully  $^1\text{H}$ -coupled  $^{13}\text{C}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate (3). This has been confirmed by unambiguous syntheses of both structures (1) and (2).





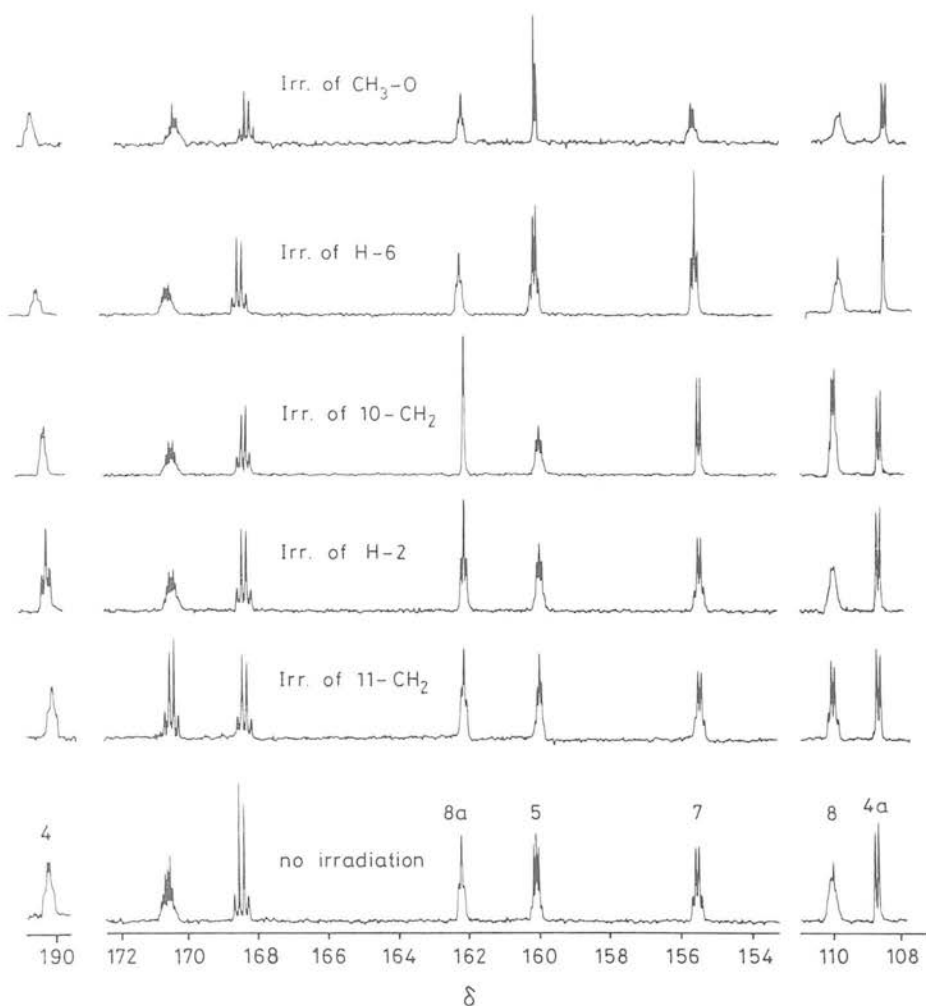


Figure 1. The high-frequency region of the fully  $^1\text{H}$ -coupled 200 MHz  $^{13}\text{C}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate, and results of selective low-power  $^1\text{H}$  decoupling experiments.

Figure 1 shows the high frequency region of the fully  $^1\text{H}$ -coupled  $^{13}\text{C}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate (3) and the results of a series of specific low-power decoupling experiments.<sup>†</sup> All the aromatic carbons give characteristic multiplet patterns.<sup>†</sup> The hydroxyethyl side chain must be placed at C-8 rather than C-6 of the chromanone skeleton as irradiation of the benzylic methylene hydrogens (10- $\text{CH}_2$ ) and H-2 respectively caused the multiplet resonance at  $\delta$  162.1 to sharpen to a broad singlet and a sharp triplet (Figure 1). These results, along with the chemical shift, indicate that this resonance must therefore be assigned to C-8a, showing 3-bond couplings to 10- $\text{CH}_2$  and H-2. Similarly the phenolic acetate must be placed at C-7 as irradiation of 10- $\text{CH}_2$  and H-6 respectively change the quartet at  $\delta$  155.1 to a doublet and a triplet; and the methoxy substituent must be placed at C-5 as the quintet at  $\delta$  160.0 collapses to a quartet and doublet respectively on irradiation of H-6 and  $\text{OCH}_3$ . The complex multiplet at  $\delta$  110.0 is assigned to C-8 as it sharpens to a quartet on irradiation of either 10- $\text{CH}_2$  or 11- $\text{CH}_2$ . It also sharpens slightly and shows an intensity increase on irradiation of H-6 due to removal of the expected 3-bond coupling. C-4a also shows a 3-bond coupling to H-6. The carbon-hydrogen couplings revealed by these experiments are sum-

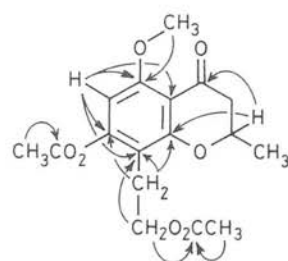
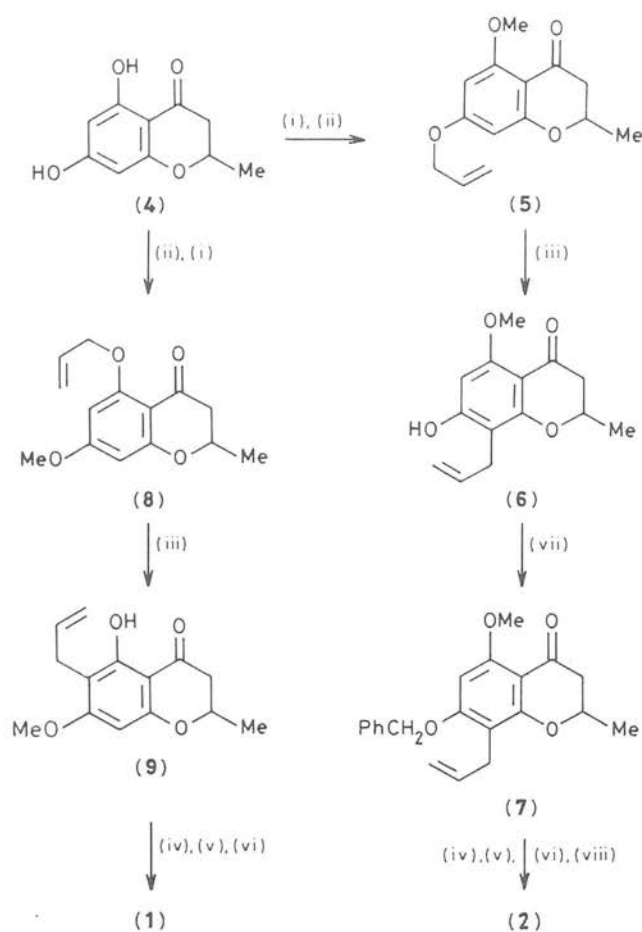


Figure 2. Long range  $^1\text{H}$ - $^{13}\text{C}$  couplings in LL-D253 $\alpha$  diacetate selectively removed by low-power  $^1\text{H}$  decoupling experiments.

marised in Figure 2. Although these effectively define structure (2) for LL-D253 $\alpha$ , confirmatory evidence was provided by unambiguous syntheses of both (1) and (2) from 5,7-dihydroxy-2-methylchromanone (4)<sup>5</sup> as outlined in Scheme 1. Allylation of the non-chelated hydroxy group on C-7 followed by methylation gave (5), which on heating to melting underwent a Claisen rearrangement to furnish (6).<sup>‡</sup> In

<sup>†</sup> C-6 gives a doublet [ $J(^{13}\text{C}$ - $^1\text{H})$  126 Hz] and exhibits no long-range couplings.

<sup>‡</sup> There is much precedent in the literature for Claisen rearrangement of 7-allyl ethers in chromone and chromanone systems to give mainly or exclusively the 8-allyl derivatives.<sup>6</sup>



**Scheme 1.** Reagents and conditions: (i)  $\text{H}_2\text{C}=\text{CHCH}_2\text{Br}$ ,  $\text{K}_2\text{CO}_3$ ; (ii)  $\text{MeI}$ ,  $\text{K}_2\text{CO}_3$ ; (iii) heat; (iv)  $\text{OsO}_4$ ; (v)  $\text{NaIO}_4$ ; (vi)  $\text{NaBH}_4$ ; (vii)  $\text{PhCH}_2\text{Br}$ ,  $\text{K}_2\text{CO}_3$ ; (viii)  $\text{H}_2$ ,  $\text{Pd}(\text{C})$ .

the presence of the free 7-hydroxy group all attempts to cleave the double bond oxidatively gave intractable mixtures. However, after protection as the benzyl ether (7), osmium tetroxide oxidation to the vicinal diol, periodate cleavage, and borohydride reduction of the resultant aldehyde, compound (2) was obtained in acceptable overall yield [16% from (4)] on hydrogenolysis of the benzyl protecting group. This was identical in all respects to natural LL-D253 $\alpha$ . Structure (1) was obtained from (4) by methylation followed by allylation to give (8), which underwent Claisen rearrangement to (9). The allyl moiety was cleaved to the hydroxyethyl moiety as above without protection of the chelated hydroxy group being necessary. The overall yield of (1) from (4) was 44%. In contrast to LL-D253 $\alpha$ , compound (1) was readily soluble in  $\text{CDCl}_3$  and its  $^1\text{H}$  n.m.r. spectrum showed the presence of a chelated hydroxy proton at  $\delta$  12.17, whereas the  $^1\text{H}$  n.m.r. spectrum of LL-D253 $\alpha$  in  $\text{CDCl}_3$  showed that no chelated hydroxy group was present.

The  $^1\text{H}$ -coupled  $^{13}\text{C}$  n.m.r. spectra of (1) and a third isomer have been obtained and will be reported in full elsewhere.

Received, 5th March 1984; Com. 295

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## Biosynthesis of LL-D253 $\alpha$ in *Phoma pigmentivora*. Incorporation of $^{13}\text{C}$ , $^2\text{H}$ , and $^{18}\text{O}$ Enriched Precursors

C. Rupert McIntyre,<sup>a</sup> Thomas J. Simpson,<sup>\*a</sup> L. A. Trimble,<sup>b</sup> and John C. Vederas<sup>b</sup>

<sup>a</sup> Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland, U.K.

<sup>b</sup> Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

The incorporation of  $^{13}\text{C}$ ,  $^2\text{H}$ , and  $^{18}\text{O}$  labelled acetates and  $^{18}\text{O}_2$  gas into LL-D253 $\alpha$  (**1**), a chromanone metabolite of *Phoma pigmentivora*, and analyses of the enriched metabolites by  $^{13}\text{C}$  and  $^2\text{H}$  n.m.r. and mass spectroscopy indicate its formation from two preformed polyketide chains; evidence for the mechanism of formation of the chromanone ring is presented, and a cyclopropyl intermediate is proposed to account for the unique randomisation of label observed in the hydroxyethyl side chain.

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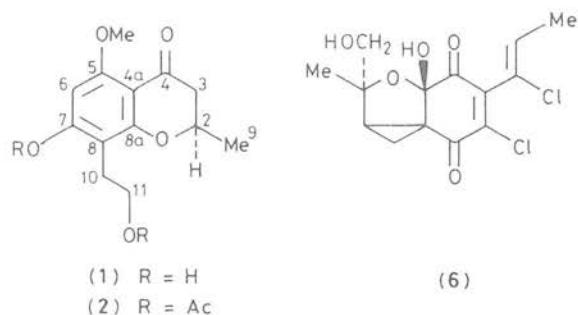
<sup>b</sup> Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

The incorporation of  $^{13}\text{C}$ ,  $^2\text{H}$ , and  $^{18}\text{O}$  labelled acetates and  $^{18}\text{O}_2$  gas into LL-D253 $\alpha$  (**1**), a chromanone metabolite of *Phoma pigmentivora*, and analyses of the enriched metabolites by  $^{13}\text{C}$  and  $^2\text{H}$  n.m.r. and mass spectroscopy indicate its formation from two preformed polyketide chains; evidence for the mechanism of formation of the chromanone ring is presented, and a cyclopropyl intermediate is proposed to account for the unique randomisation of label observed in the hydroxyethyl side chain.

LL-D253 $\alpha$  (**1**), a metabolite of *Phoma pigmentivora*, is a chromanone with an unusual hydroxyethyl substituent.<sup>1</sup> Structural analysis strongly suggests a polyketide derivation for the chromanone nucleus but the origin of the  $\text{C}_2$ -side chain is obscure. Possible routes include: (a) condensation of two preformed polyketide chains;<sup>2</sup> or elaboration of the side chain onto a preformed pentaketide-derived precursor either by (b)

C-acylation,<sup>3</sup> or (c) stepwise introduction of two  $\text{C}_1$  equivalents,<sup>4</sup> or (d) introduction and oxidative cleavage of a prenyl substituent.<sup>5</sup> Since all of these represent unusual routes in fungal polyketide biosynthesis, we have carried out incorporation studies with  $^{13}\text{C}$ ,  $^2\text{H}$ , and  $^{18}\text{O}$  labelled acetates and  $^{18}\text{O}_2$  to identify the correct pathway.

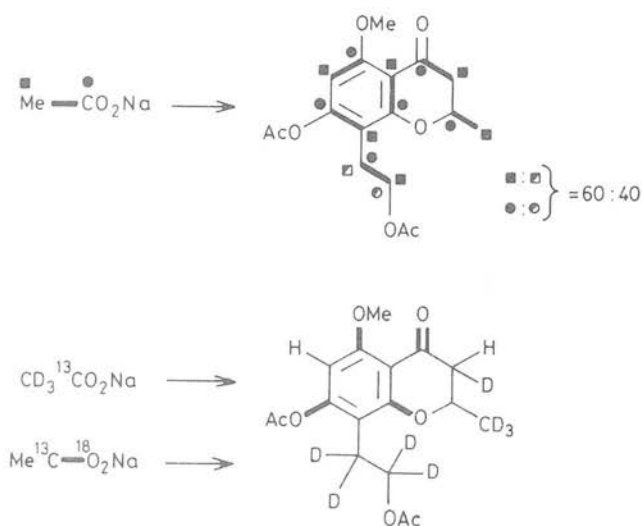
Incorporation of [ $^{13}\text{C}_2$ ]acetate indicated that all the skeletal



**Table 1.**  $^{18}\text{O}$  Isotopically shifted resonances observed in the 100.6 MHz  $^{13}\text{C}$  n.m.r. spectrum of LL-D253 $\alpha$  diacetate (2).

Carbon	$\delta$ (p.p.m.)	$\Delta\delta$ (p.p.m. $\times 100$ )	Ratio $^{16}\text{O} : ^{18}\text{O}^c$
4	190.1 <sup>a</sup>	4.1	— (90 : 8)
8a	162.1 <sup>a</sup>	1.6	80 : 20 (80 : 20)
5	160.0 <sup>a</sup>	1.7	78 : 22 (75 : 25)
7	155.1 <sup>a</sup>	2.1	84 : 16 (83 : 17)
11	62.8 <sup>b</sup>	2.7	86 : 14 (85 : 15)

<sup>a</sup>  $[1-^{13}\text{C}, ^{18}\text{O}_2]$ Acetate-enriched. <sup>b</sup>  $^{18}\text{O}_2$ -Enriched. <sup>c</sup> Values in parentheses are from DuPont Curve Resolver.



**Scheme 1**

carbons were derived from intact acetate units as shown in Scheme 1. It is noteworthy that no randomisation of labelling was observed in the phloroglucinol ring suggesting that it cannot have been symmetrically substituted at any point in the biosynthetic pathway. The results of incorporating  $[1-^{13}\text{C}]$ - and  $[2-^{13}\text{C}]$ -acetates are particularly interesting. As anticipated C-2, C-4, C-5, C-7, C-8a, and C-10 were all highly enriched by  $[1-^{13}\text{C}]$ acetate. However C-11 also showed significant enrichment, the combined enrichment at C-10 and C-11, and that at C-2 being significantly higher than that at the other enriched sites. Analogous results were obtained with  $[2-^{13}\text{C}]$ acetate but now C-11 was more highly enriched than C-10.<sup>†</sup> Incorporation of label from  $[5-^{14}\text{C}]$ mevalonic acid was negligible thereby rendering route (d), our initially favoured pathway, unlikely.

The fate of acetate-derived hydrogen can be studied by incorporation of  $[1-^{13}\text{C}, ^2\text{H}_3]$ acetate and detection of deuterium-induced  $\beta$ -isotope shifts in the  $^{13}\text{C}$  n.m.r. spectrum of the enriched metabolite.<sup>6,7</sup> Figure 1 shows the  $^{13}\text{C}$  n.m.r. spectrum of  $[1-^{13}\text{C}, ^2\text{H}_3]$ acetate-enriched LL-D253 $\alpha$  diacetate (2). Thus C-2 shows isotopically shifted resonances corresponding to the incorporation of one, two, and (mainly) three deuterium atoms at C-9, indicating its origin from an acetate

'starter' unit;<sup>8</sup> C-4 shows one downfield shifted resonance,<sup>7</sup> corresponding to the incorporation of one deuterium atom at C-3; and finally both C-10 and C-11 each showed two isotopically shifted resonances corresponding to the incorporation of two deuterium atoms at C-11 and C-10 respectively. All these results are summarised in Scheme 1.

On incorporation of  $[1-^{13}\text{C}, ^{18}\text{O}_2]$ acetate, the  $^{13}\text{C}$  n.m.r. spectrum of (2) showed isotopically shifted resonances for C-4, -5, -7, and -8a, indicating that the oxygens attached to these carbons are acetate-derived, and therefore that the corresponding carbon-oxygen bonds had remained intact throughout the course of the biosynthesis (Table 1).<sup>9</sup> These results and the incorporation of only one deuterium at C-3, indicate that the chromanone ring is formed by conjugate addition of a phenolic hydroxy group to the corresponding  $\alpha,\beta$ -unsaturated ketone. LL-D253 $\alpha$  has the 2R configuration<sup>10</sup> so the ring closure process is stereospecific with respect to C-2. To examine the stereospecificity with respect to C-3 the  $^2\text{H}$  n.m.r. spectrum of  $[^2\text{H}_3]$ acetate-enriched LL-D253 $\alpha$  diacetate was examined. The axial and equatorial hydrogens at C-3 have almost coincident chemical shifts and even at high field strengths form part of a complex ABX system. However on addition of  $\text{Eu}(\text{fod})_3$  (fod = 1,1,1,2,2,3,3,3-heptafluoro-7,7-dimethyloctane-4,6-dionate), the induced shifts in the 360 MHz  $^1\text{H}$  n.m.r. spectrum allow the resonances to be resolved and assigned from their coupling constants to the axial and equatorial hydrogens. Determining the  $^2\text{H}$  n.m.r. spectrum of  $[\text{U}-^2\text{H}]$ -LL-D253 $\alpha$ <sup>‡</sup> under the same conditions confirmed that the corresponding  $^2\text{H}$  resonances could also be resolved. Finally repeating the experiment with the  $[^2\text{H}_3]$ acetate-enriched compound showed that both positions were enriched to equal extents so that protonation of the intermediate enolate must proceed with equal facility from both sides of the molecule (Scheme 2). This contrasts with the corresponding chalcone to flavanone ring closure which has been shown to be completely stereospecific.<sup>11</sup>

The observed randomisation of label between C-10 and C-11 indicates that these carbons may become equivalent during the biosynthesis. To account for this and the lack of randomisation of label in the phloroglucinol ring we propose the pathway shown in Scheme 3. Two preformed polyketide chains, either a  $\text{C}_4$  plus  $\text{C}_8$  as shown, or  $\text{C}_6$  plus  $\text{C}_6$ , or  $\text{C}_2$  plus  $\text{C}_{10}$ , probably condense before aromatisation of ring A. The transposition of oxygen in the  $\text{C}_2$ -side chain and randomisation of label can then be explained by reduction and elimination (*cf.* fatty acid biosynthesis) to give the vinyl

<sup>†</sup> The relative enrichments at C-10 and C-11 are consistent with label from C-1 or C-2 of acetate being randomised to the extent of *ca.* 80% between C-10 and C-11, *e.g.* in the  $[1-^{13}\text{C}]$ acetate feed C-10 has 60% of the label and C-11 40%.

<sup>‡</sup> Prepared by fermentation of *P. pigmentivora* in a medium supplemented with 5%  $^2\text{H}_2\text{O}$ .<sup>13</sup>

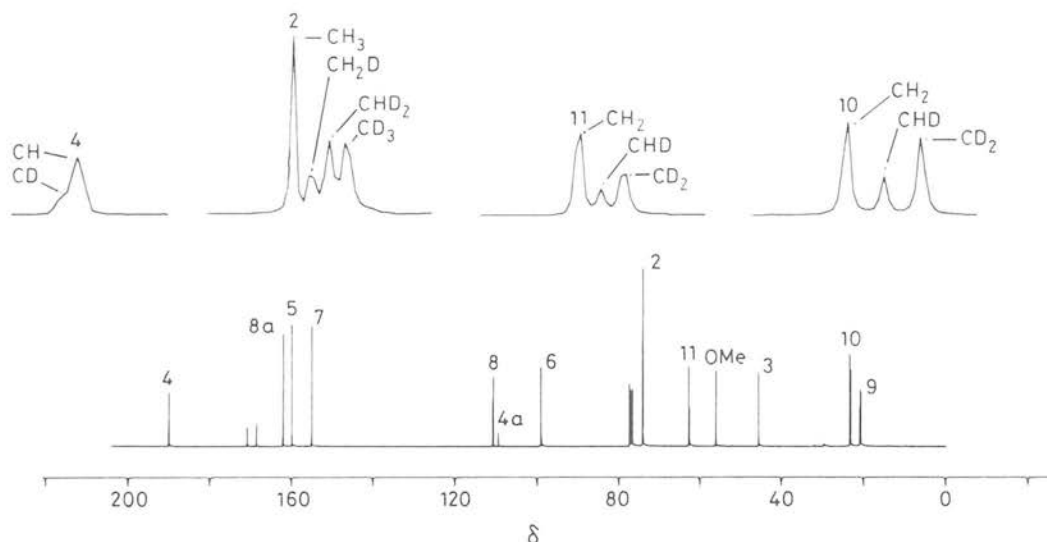
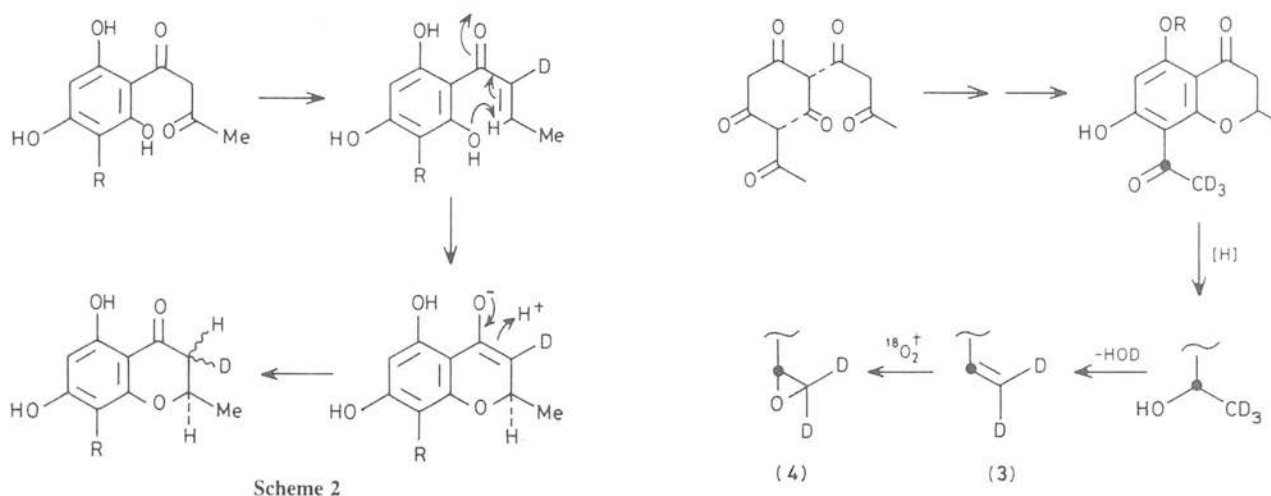
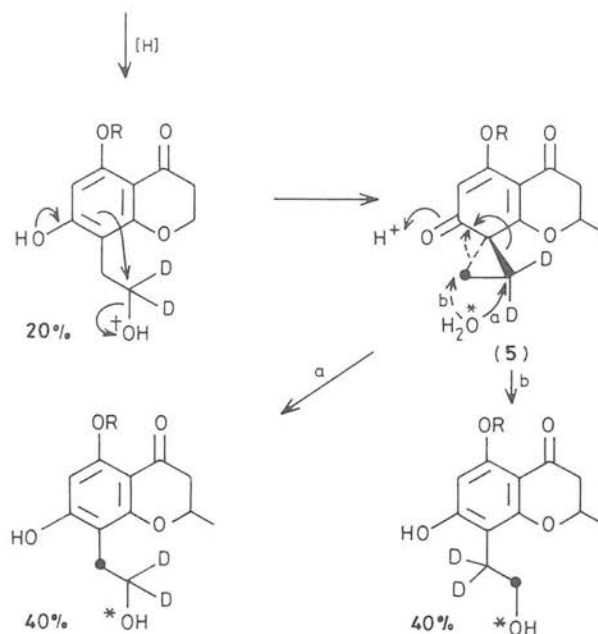


Figure 1. 90.56 MHz Proton noise-decoupled  $^{13}\text{C}$  n.m.r. spectrum of  $[1\text{-}^{13}\text{C}, 2\text{H}_3]$ acetate-enriched LL-D253 $\alpha$  diacetate in  $\text{CDCl}_3$ .



intermediate (3). Epoxidation and reductive opening of the epoxide (4) would furnish the hydroxyethyl moiety directly. To account for the observed 80% randomisation of label between C-10 and C-11 we propose the involvement of a cyclopropyl intermediate (5) which would be formed by participation of the phloroglucinol ring in expulsion of the hydroxy group at C-11. Hydrolytic opening of the resulting cyclopropyl ring at either the  $\alpha$  or  $\beta$  carbons would then produce the observed randomisation of labelling between C-10 and C-11 in (1). An analogy for this process is found in the mould metabolite mikrolin (6) which contains a cyclopropyl ring fused to a cyclohexadienone moiety.<sup>12</sup> The observed degree of randomisation requires that 20% of the natural product is derived directly from reduction of the epoxide (4). Support for this was provided by carrying out a fermentation of *P. pigmentivora* under an atmosphere of  $^{18}\text{O}_2$ . The mass spectrum of the derived LL-D253 $\alpha$  shows an  $M + 2$  peak, corresponding to 15% of the metabolite, which mass matches for  $\text{C}_{13}\text{H}_{16}^{16}\text{O}_4^{18}\text{O}$ . In addition, examination of the  $^{13}\text{C}$  n.m.r. spectrum for isotope shifts determined that, within experimental error, all of the  $^{18}\text{O}$  was located at C-11 (Table 1). At present we cannot say with certainty whether the randomisation is an *in vitro* or *in vivo* process. However  $[10\text{-}^2\text{H}_2]$ -LL-D253 $\alpha$  has been prepared and no randomisation



of label between C-10 and C-11 is observed on either mild acid or mild base treatment.

We thank the S.E.R.C., NATO, and the Natural Sciences and Engineering Research Council of Canada (NSERC) for financial support.

Received, 5th March 1984; Com. 296

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## Biosynthesis of the Meroterpenoid Metabolite, Andibenin B: Incorporation of Sodium [1- $^{13}\text{C}$ , $^{18}\text{O}_2$ ]Acetate and $^{18}\text{O}_2$

C. Rupert McIntyre,<sup>a</sup> Thomas J. Simpson,<sup>\*a</sup> Richard N. Moore,<sup>b</sup> Laird A. Trimble,<sup>b</sup> and John C. Vederas<sup>\*b</sup>

<sup>a</sup> Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland, U.K.

<sup>b</sup> Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

Incorporation of sodium [1- $^{13}\text{C}$ ,  $^{18}\text{O}_2$ ]acetate and  $^{18}\text{O}_2$  gas into andibenin B (1) by cultures of *Aspergillus variegator* and observation of  $^{18}\text{O}$  isotope-induced shifts in the  $^{13}\text{C}$  n.m.r. spectra of the enriched metabolites establish the origins of all the oxygen atoms and provide mechanistic information on the biosynthetic pathway.

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<sup>a</sup> Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland, U.K.

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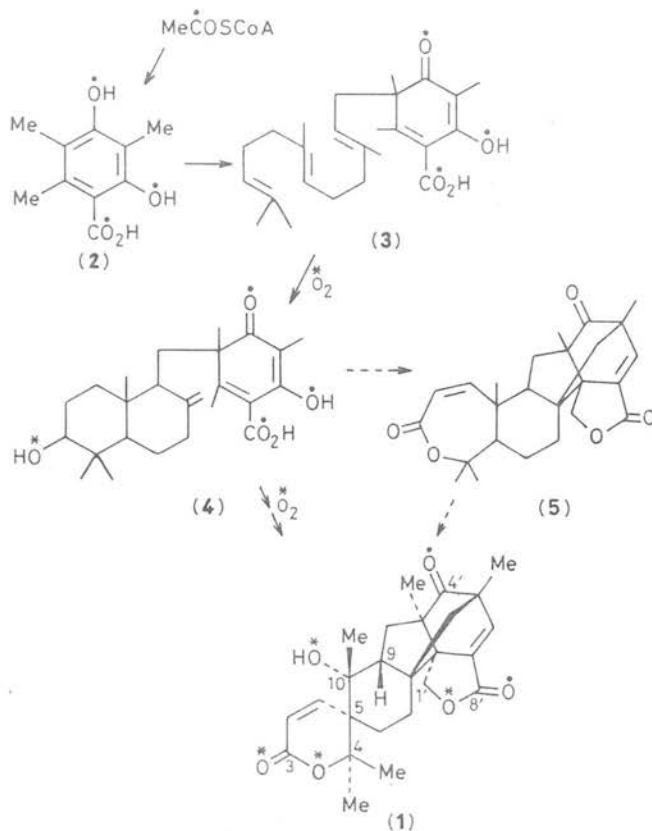
Incorporation of sodium [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate and <sup>18</sup>O<sub>2</sub> gas into andibenin B (1) by cultures of *Aspergillus variegator* and observation of <sup>18</sup>O isotope-induced shifts in the <sup>13</sup>C n.m.r. spectra of the enriched metabolites establish the origins of all the oxygen atoms and provide mechanistic information on the biosynthetic pathway.

We have recently shown that andibenin B (1), a metabolite of *Aspergillus variegator*, is a member of a group of biosynthetically related metabolites formed by a mixed polyketide-terpenoid pathway.<sup>1,2</sup> The key step is alkylation of 3,5-dimethylorsellinic acid (2), a bis-C-methylated tetraketide, with farnesyl pyrophosphate to give (3), which then cyclises to give product (4). Further condensations and oxidative modifications convert (4) into andibenin B (1). Since observation of isotope shifts induced in <sup>13</sup>C n.m.r. spectra by <sup>18</sup>O provides information on intermediate oxidation states and mechanisms,<sup>3</sup> we have incorporated sodium [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate and <sup>18</sup>O<sub>2</sub> gas into andibenin B (1) to study these modifications.

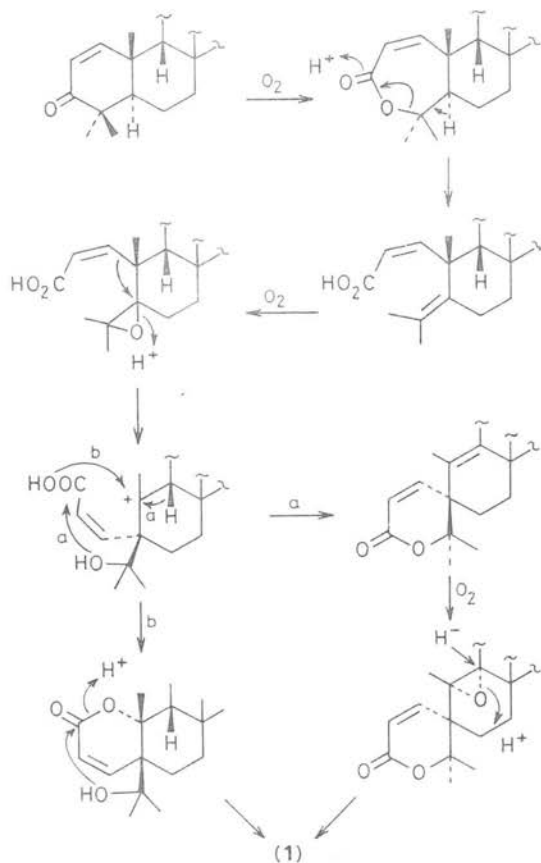
The proton noise-decoupled <sup>13</sup>C n.m.r. spectrum of (1) enriched by fermentation of *A. variegator* with sodium [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate shows isotopically shifted signals (Table 1) due to incorporation of acetate-derived oxygen into the C-4' and C-8' carbonyl groups. In a separate experiment, growth of the cultures under an <sup>18</sup>O<sub>2</sub> atmosphere with unlabelled carbon sources produced andibenin B (1) whose <sup>13</sup>C n.m.r. spectrum demonstrated the origin of both oxygen atoms on C-3 and of the single-bonded oxygen atoms on C-4,

C-10, C-1', and C-8' from oxidative processes. The appearance of oxygen label in the γ-lactone ring oxygen in the <sup>18</sup>O<sub>2</sub> experiment suggests that the pathway proceeds by hydroxylation of the 6-methyl group of (2) followed by nucleophilic attack of this hydroxy group on the carboxy group. It remains to be established whether ring closure occurs before or after alkylation with farnesyl pyrophosphate.

In accord with earlier carbon labelling studies,<sup>4</sup> the present results show that the C-3 lactone function must be formed by a biological Baeyer-Villiger-type oxidation of a corresponding ketone precursor. Generation of the spiro ring system involves a ring contraction which requires development of formal carbocation character at C-5. Similar ring contractions have been observed in steroid derivatives on acid treatment of either 4β,5β- or 5α,6α-epoxides.<sup>6</sup> Since the 10-hydroxy group of andibenin B (1) is derived from atmospheric oxygen, the rearrangement cannot terminate by capture of an intermediate C-10 carbocation by water. Instead, intramolecular attack by the carboxy group or an elimination-epoxidation-



Scheme 1



Scheme 2

Table 1.  $^{18}\text{O}$  Isotopically shifted resonances observed in the 100.6 MHz  $^{13}\text{C}$  n.m.r. spectrum of andibenin B (1).<sup>a</sup>

Carbon	$\delta$ (p.p.m.)	$\Delta\delta$ (p.p.m. $\times 100$ )
4 <sup>c</sup>	215.8	5.0 <sup>b</sup>
8 <sup>c</sup>	169.0	3.5 <sup>b</sup>
		1.2 <sup>c</sup>
3	165.5	4.7 <sup>c</sup>
4	86.9	4.2 <sup>c</sup>
10	79.3	3.2 <sup>c</sup>
1 <sup>c</sup>	70.4	2.9 <sup>c</sup>

<sup>a</sup> For experimental conditions see ref. 9. <sup>b</sup> Enriched by sodium [ $1\text{-}^{13}\text{C}, ^{18}\text{O}_2$ ]acetate. <sup>c</sup> Enriched by  $^{18}\text{O}_2$ .

reduction sequence as shown in Scheme 2 is probably responsible for the hydroxy function. The closely related metabolite austin<sup>2</sup> contains the 9,10 double bond which would be present in the intermediate of the latter pathway. The co-occurrence of andilesin B (5) with andibenin B (1)<sup>7</sup> suggests that ring contraction follows lactone formation. Steroidal  $\epsilon$ -lactones are known to undergo similar facile ring opening under acidic conditions.<sup>8</sup>

We thank the S.E.R.C., N.A.T.O., and the Natural Sciences and Engineering Research Council of Canada (N.S.E.R.C.) for financial support.

Received, 18th June 1984; Com. 852

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**Biosynthesis of Terretonin, a Polyketide-terpenoid Metabolite  
of *Aspergillus terreus***

By C. RUPERT MCINTYRE and THOMAS J. SIMPSON\*

(Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland)

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Chemical Communications 1981**

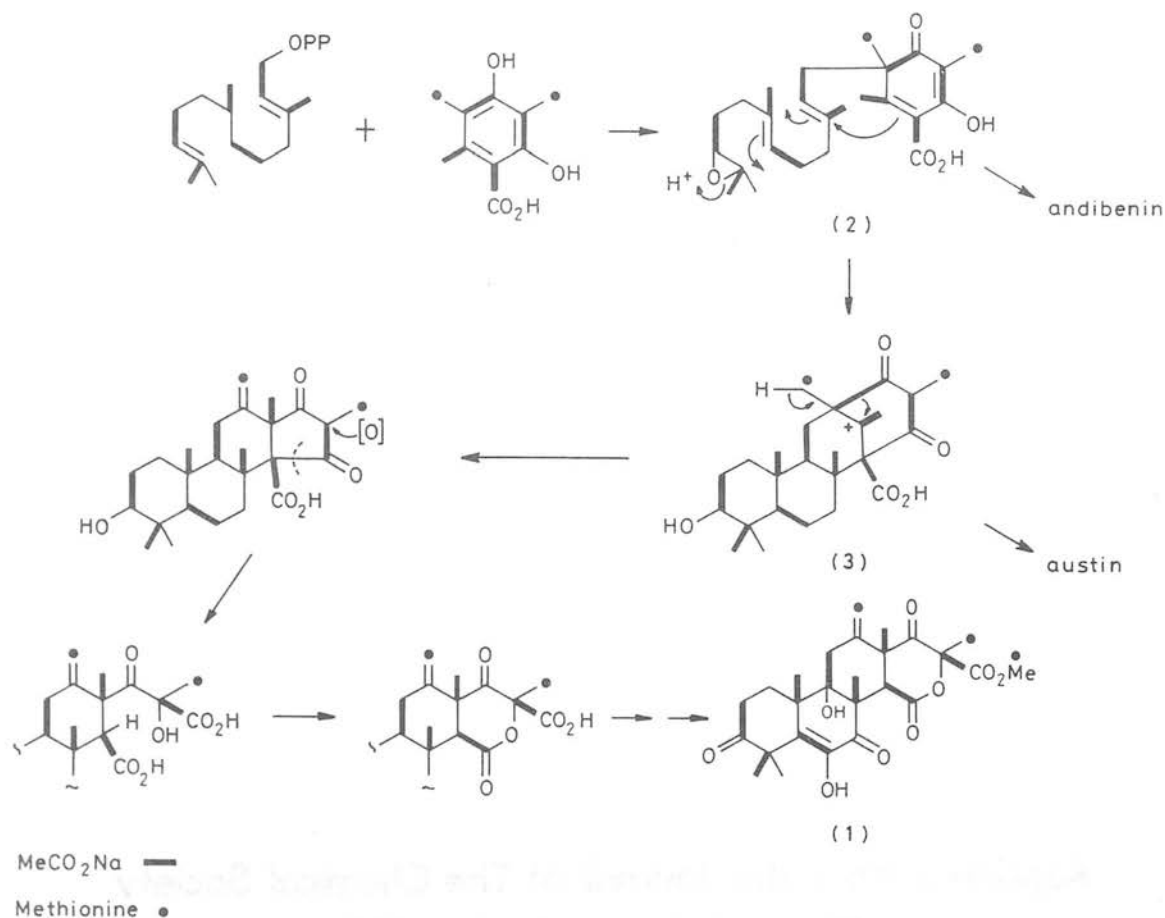
## Biosynthesis of Terretonin, a Polyketide-terpenoid Metabolite of *Aspergillus terreus*

By C. RUPERT McINTYRE and THOMAS J. SIMPSON\*

(Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland)

**Summary** Incorporations of [1,2- $^{13}\text{C}_2$ ]acetate and [Me- $^{13}\text{C}$ ]-methionine into the mycotoxin terretonin, a metabolite of *Aspergillus terreus*, indicate its formation by a mixed polyketide-terpenoid pathway.

ANDIBENIN<sup>1</sup> and anditomin,<sup>2</sup> metabolites of *Aspergillus variegatus*, and austin,<sup>3</sup> a metabolite of *Aspergillus ustus*, have been shown to be formed by a novel variation of the triphenyl-phenol biosynthetic pathway in which C-



SCHEME. Biosynthesis of terretonin in *Aspergillus terreus*

alkylation of 3,5-dimethylorsellinate by farnesyl pyrophosphate gives the key intermediate (2) which is then subject to further elaboration to produce the above metabolites. We suggested<sup>2</sup> that the mycotoxin terretonin (1), a metabolite of *Aspergillus terreus* for which a triterpenoid origin has been proposed,<sup>4</sup> could also be a product of this pathway. We now report studies which support this proposal.

Incorporation of [*Me*-<sup>13</sup>C]methionine and [1,2-<sup>13</sup>C<sub>2</sub>]acetate by cultures of *A. terreus* (NRRL 6273) resulted in terretonin being labelled as indicated in the Scheme.† This labelling pattern can be best accounted for by the pathway sum-

marised in the Scheme in which cyclisation of (2) as in austin biosynthesis gives the intermediate (3), which by ring contraction, followed by retro-Claisen reaction, hydroxylation, and lactonisation as indicated to generate the ring D keto-lactone system, and oxidative modification of the bicyclofarnesyl moiety, gives terretonin. Further studies to establish the timing and mechanisms of these processes are in progress.

The support of the S.R.C. and microbiological assistance by Miss S. Christie are gratefully acknowledged.

(Received, 1st July 1981; Com. 772.)

† Details of <sup>13</sup>C n.m.r. assignments, enrichments, and <sup>13</sup>C-<sup>13</sup>C couplings will be reported in full elsewhere.

<sup>1</sup> J. S. E. Holker and T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1978, 626.

<sup>2</sup> T. J. Simpson, *Tetrahedron Lett.*, in the press.

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## Biosynthesis of the Meroterpenoid Metabolites, Austin and Terretonin: Incorporation of 3,5-Dimethylorsellinate

C. Rupert McIntyre,<sup>a</sup> Thomas J. Simpson,<sup>\*a</sup> Desmond J. Stenzel,<sup>a</sup> Alan J. Bartlett,<sup>b</sup> Eugene O'Brien,<sup>b</sup> and John S. E. Holker<sup>b</sup>

<sup>a</sup> Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, U.K.

<sup>b</sup> Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

<sup>14</sup>C and <sup>2</sup>H Labelling experiments, together with <sup>2</sup>H n.m.r. spectroscopy show that 3,5-dimethylorsellinic acid is a specific precursor of austin and terretonin in *Aspergillus ustus* and *Aspergillus terreus*, respectively, and so substantiate the mixed polyketide-terpenoid origin proposed for these metabolites from the incorporation of <sup>13</sup>C-labelled simple precursors.

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Chemical Communications 1982

## Biosynthesis of the Meroterpenoid Metabolites, Austin and Terretonin: Incorporation of 3,5-Dimethylorsellinate

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<sup>a</sup> Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, U.K.

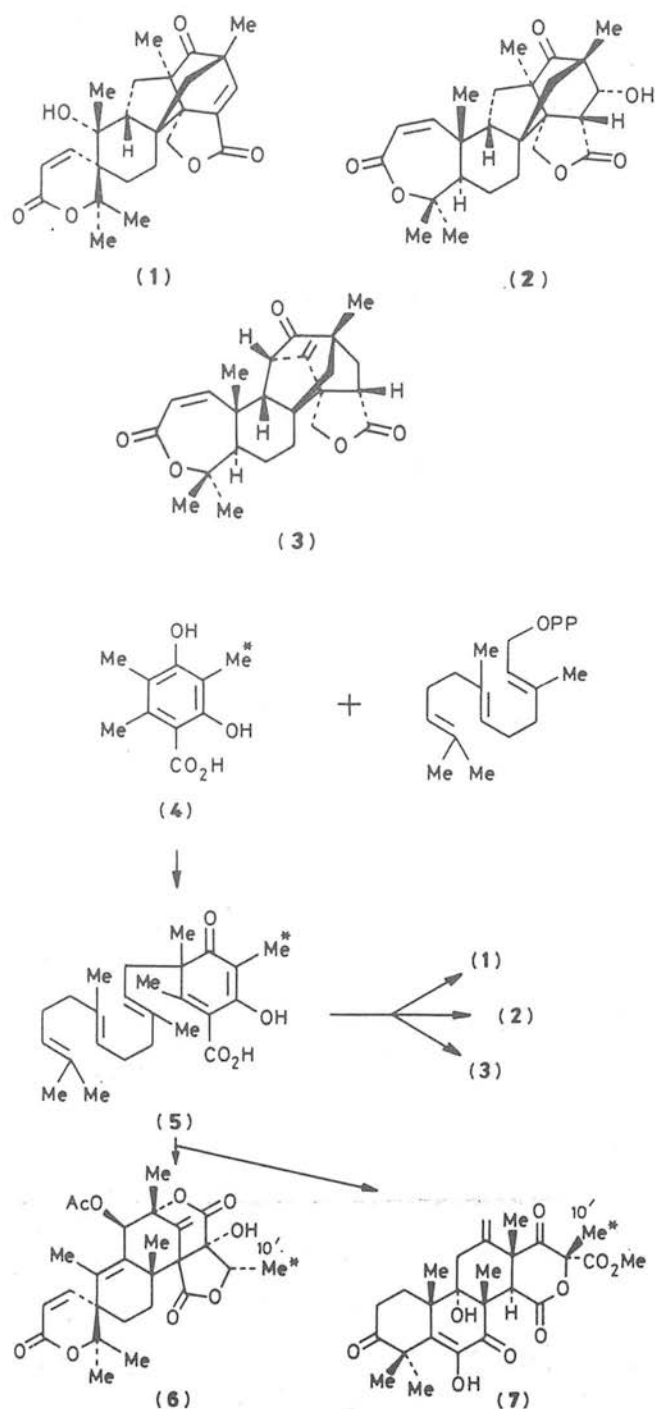
<sup>b</sup> Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

<sup>14</sup>C and <sup>2</sup>H Labelling experiments, together with <sup>2</sup>H n.m.r. spectroscopy show that 3,5-dimethylorsellinic acid is a specific precursor of austin and terretonin in *Aspergillus ustus* and *Aspergillus terreus*, respectively, and so substantiate the mixed polyketide-terpenoid origin proposed for these metabolites from the incorporation of <sup>13</sup>C-labelled simple precursors.

<sup>13</sup>C and <sup>2</sup>H Labelling studies<sup>1-3</sup> have shown that andibenin B (1), andilesin A (2), and anditomin (3), C<sub>25</sub> metabolites of *Aspergillus variegator* are formed by a mixed polyketide-terpenoid biosynthetic pathway in which the key step is alkylation of 3,5-dimethylorsellinic acid (4) by farnesyl pyrophosphate to give (5). We suggested that the mycotoxins austin (6) and terretonin (7), metabolites of *Aspergillus ustus* and *Aspergillus terreus*, respectively, could also be formed via intermediate (5), and incorporations of singly and doubly

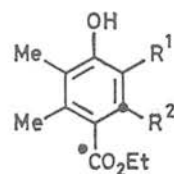
labelled [<sup>13</sup>C]acetates and [<sup>13</sup>C]methionine into austin and terretonin result in labelling patterns consistent with these proposals.<sup>4,5</sup> However, as the suggested pathways require unprecedented degrees of modification<sup>6</sup> of the tetraketide-derived phenolic precursor, we have carried out and now report studies to show that 3,5-dimethylorsellinate is indeed a specific precursor of both austin and terretonin (Scheme 1).

Ethyl [carboxy,2-<sup>14</sup>C<sub>2</sub>]-3,5-dimethylorsellinate (8) (39.7  $\mu$ Ci mmol<sup>-1</sup>)<sup>2</sup> was fed to static cultures of *A. ustus* (24 mg to

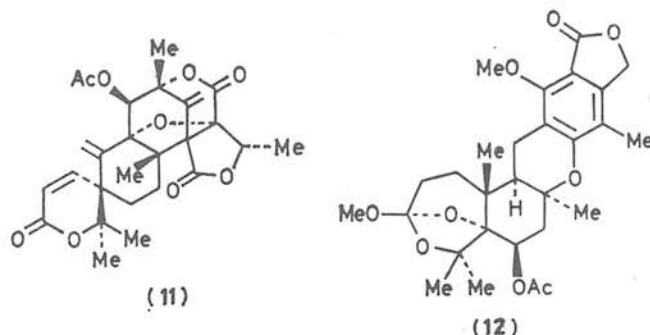


Scheme 1

0.41) and *A. terreus* (33 mg to 1 l), respectively, to give austin (36 mg,  $1.60 \mu\text{Ci mmol}^{-1}$ ) and terretonin (34 mg,  $1.01 \mu\text{Ci mmol}^{-1}$ ), specific incorporations of 4.0 and 2.5%, respectively. Interestingly, the 2-deoxyorsellinate (9) which was incorporated<sup>3</sup> into andibenin B with comparable efficiency to (8) was not incorporated into austin to any significant extent (specific incorporation, 0.06%). As with andibenin B, the complexity of the metabolites precluded the degradative studies essential to establish specificity of labelling, so the trideuteriomethyl analogue (10)<sup>3</sup> was fed, and the resultant enriched metabolites analysed by 55 MHz  $^3\text{H}$  n.m.r. spectroscopy. Austin showed only one signal at  $\delta$  1.22 p.p.m.; terretonin similarly showed only one signal at  $\delta$  1.68 p.p.m.,



- (8)  $\text{R}^1 = \text{CH}_3$ ,  $\text{R}^2 = \text{OH}$ ,  $\bullet = {}^{14}\text{C}$   
 (9)  $\text{R}^1 = \text{CH}_3$ ,  $\text{R}^2 = \text{H}$ ,  $\bullet = {}^{14}\text{C}$   
 (10)  $\text{R}^1 = \text{CD}_3$ ,  $\text{R}^2 = \text{OH}$ ,  $\bullet = {}^{12}\text{C}$



chemical shifts corresponding in each metabolite to the 10'-methyl hydrogens,<sup>†</sup> in agreement with our proposed pathways.<sup>4,5</sup> Thus it is clear that 3,5-dimethylorsellinate is a specific precursor to both austin and terretonin and so their meroterpenoid<sup>7</sup> origins are established beyond doubt.

Further evidence for the common biogenetic origins of these metabolites comes from the isolation of austin and dehydroaustin (11), a co-metabolite of austin in *A. ustus*,<sup>8</sup> from a chance mutant of the andibenin producing culture of *A. variegata* which no longer produced andibenin. Another observation to note is the recent isolation of the austerolides [e.g. (12)] from a toxigenic strain of *A. ustus*.<sup>9</sup> Structural analysis suggests they are biosynthesised *via* alkylation of 5-methylorsellinic acid by farnesyl pyrophosphate, *cf.* mycophenolic acid,<sup>10</sup> followed by cyclisation of the farnesyl moiety and oxidative modifications analogous to those occurring in the andibenins and andileins.

Received, 25th March 1982; Com. 346

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<sup>†</sup> Control experiments show that the 10'-methyl signals in the  $^3\text{H}$  n.m.r. spectra of universally deuterated austin and terretonin are sufficiently well resolved from other signals to ensure specificity of labelling.



## Biosynthesis of Tajixanthone and Shamixanthone by *Aspergillus variegator*: Incorporation of Oxygen-18 Gas

Esfandiar Bardshiri,<sup>a</sup> C. Rupert McIntyre,<sup>a</sup> Thomas J. Simpson,<sup>a\*</sup> Richard N. Moore,<sup>b</sup> Laird A. Trimble,<sup>b</sup> and John C. Vederas<sup>b\*</sup>

<sup>a</sup> Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland, U.K.

<sup>b</sup> Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

Mass spectral and <sup>13</sup>C n.m.r. analyses of tajixanthone (**1**) and shamixanthone (**2**) formed during growth of *Aspergillus variegator* under atmospheres containing [<sup>18</sup>O<sub>2</sub>] oxygen gas showed incorporation of four and three <sup>18</sup>O labels per molecule of (**1**) and (**2**), respectively, and provided information about the mode of xanthone ring formation.

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*Chemical Communications* 1984

## Biosynthesis of Tajixanthone and Shamixanthone by *Aspergillus varicolor*: Incorporation of Oxygen-18 Gas

Esfandiar Bardshiri,<sup>a</sup> C. Rupert McIntyre,<sup>a</sup> Thomas J. Simpson,<sup>a\*</sup> Richard N. Moore,<sup>b</sup> Laird A. Trimble,<sup>b</sup> and John C. Vederas<sup>b\*</sup>

<sup>a</sup> Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland, U.K.

<sup>b</sup> Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

Mass spectral and <sup>13</sup>C n.m.r. analyses of tajixanthone (1) and shamixanthone (2) formed during growth of *Aspergillus varicolor* under atmospheres containing [<sup>18</sup>O<sub>2</sub>] oxygen gas showed incorporation of four and three <sup>18</sup>O labels per molecule of (1) and (2), respectively, and provided information about the mode of xanthone ring formation.

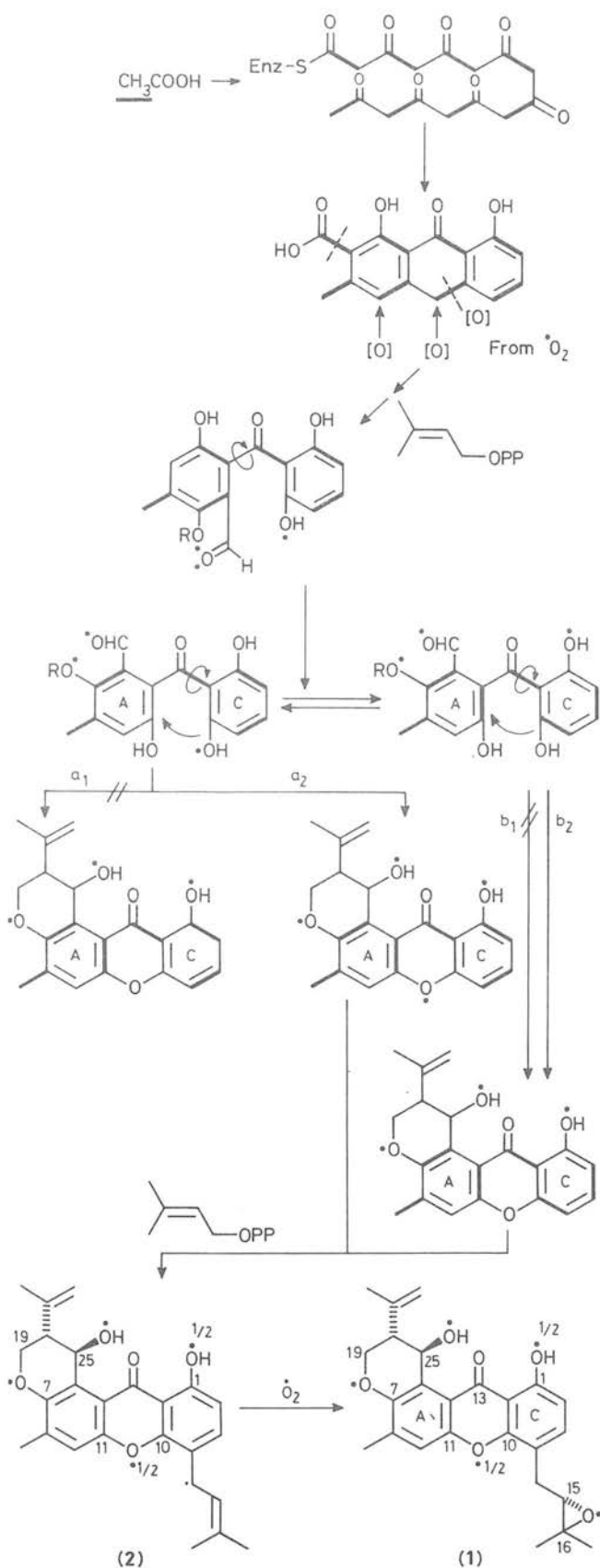
Mycelial pigments like tajixanthone (1) and shamixanthone (2)<sup>1</sup> as well as various meroterpenoids<sup>2</sup> illustrate how *Aspergillus* species can combine polyketide and terpenoid precursors to form secondary metabolites which have often undergone extensive oxidative elaboration. The isolation of a number of closely related xanthenes<sup>3–5</sup> and <sup>13</sup>C and <sup>2</sup>H labelling studies<sup>6,7</sup> on tajixanthone strongly support the

biosynthetic pathway outlined in Scheme 1. Carbon labelling results suggest that an acetate-derived octaketide precursor cyclizes to an anthrone which is hydroxylated, *O*-prenylated by dimethylallyl pyrophosphate, and oxidatively cleaved to a benzophenone derivative, either directly or after oxidation to an anthraquinone. Observation of two distinct carbon labelling patterns present in equal amounts in ring c of (1) implies

**Table 1.** <sup>18</sup>O Isotopically-shifted resonances in the <sup>13</sup>C n.m.r. spectra<sup>a</sup> of tajixanthone (1) and shamixanthone (2).

Carbon	δ (1)	Δδ (1) (× 100)	<sup>16</sup> O : <sup>18</sup> O (1) <sup>d</sup>	δ (2)	Δδ (2) (× 100)	<sup>16</sup> O : <sup>18</sup> O (2) <sup>d</sup>
13	184.0	2.7 <sup>b</sup>				
1	160.4 <sup>c</sup>	1.0	79 : 21	159.7	1.0 <sup>c</sup>	
10	152.9 <sup>c</sup>	2.3	80 : 20	152.8	2.5	71 : 29
11	152.0	2.4	77 : 23	152.2	2.3	73 : 27
7	149.5	1.5	61 : 39	149.4	1.6	53 : 47
19	64.5	2.3	66 : 34	64.5	2.5	55 : 45
15	63.24	3.3	64 : 36			
25	63.16	1.5	66 : 34	63.2	1.5	60 : 40
16	58.5	4.1	64 : 36			

<sup>a</sup> Spectra run at 100.6 and 90.6 MHz; for experimental conditions see ref. 9. <sup>b</sup> Enriched by sodium [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate only; all others enriched by <sup>18</sup>O<sub>2</sub>. <sup>c</sup> These assignments were originally reversed in ref. 6. <sup>d</sup> Approximate (±5%) ratios from peak areas. <sup>e</sup> Not resolved completely.



Scheme 1

the intermediacy of a symmetrical dihydroxyphenyl moiety which is free to rotate prior to cyclization to a xanthone.<sup>7</sup> Since the detection of <sup>18</sup>O-induced isotope shifts in <sup>13</sup>C n.m.r.<sup>8</sup> has proved useful in determining the mode of xanthone ring formation in ravenelin<sup>9</sup> and sterigmatocystin,<sup>10</sup> we have studied the incorporation of <sup>18</sup>O<sub>2</sub> gas into tajixanthone (1) and shamixanthone (2).

A fermentation of *Aspergillus varicolor*<sup>1</sup> in which the normal atmosphere was replaced with one containing <sup>18</sup>O<sub>2</sub> gas (98.7% isotopic purity) gave tajixanthone (1), the mass spectrum of which showed the presence of four <sup>18</sup>O atoms per molecule. The 100.6 and 90.6 MHz <sup>13</sup>C n.m.r. spectra of a mixture of this and unlabelled material displayed isotopically-shifted resonances for eight of the nine oxygen-bearing carbons (Table 1). Only the carbonyl oxygen at C-13 remained completely unlabelled in this experiment. Within experimental error, the relative amount of <sup>18</sup>O incorporated at C-1 and at C-10 is half of that at the other labelled sites. Taken together with the mass spectral results, this shows that in a particular molecule of tajixanthone (1) either the oxygen at C-1 or the one at C-10 was labelled, but not both. This confirms the intermediacy and oxidative origin of a conformationally labile benzophenone which has an axis of symmetry in a dihydroxyphenyl ring. More importantly, the results demonstrate that xanthone ring closure must proceed almost exclusively by a Michael addition-elimination<sup>11</sup> process in which the ring c oxygen attacks the ring a carbon with ultimate loss of the ring a oxygen at C-11 (paths a<sub>2</sub> and b<sub>2</sub>). Cyclization in the opposite sense with retention of the ring a oxygen (paths a<sub>1</sub> and b<sub>1</sub>) is very minor if it occurs at all.

The presence of <sup>18</sup>O at C-25 and the previously reported loss of <sup>2</sup>H from acetate at that position<sup>7</sup> suggest oxidative cleavage of an anthraquinone rather than anthrone precursor. Mass spectral analysis of the molecular ion region of (1) obtained from a fermentation utilizing a mixture of <sup>16</sup>O<sub>2</sub> and <sup>18</sup>O<sub>2</sub> shows that each aerobically-derived oxygen atom is introduced separately by mono-oxygenation. Thus the involvement of dioxygenase-derived dioxetanes<sup>12</sup> or endoperoxides<sup>13,14</sup> which have been proposed as intermediates in the cleavage mechanism can be ruled out. Presumably cleavage occurs *via* a biological Baeyer-Villiger type oxidation<sup>15</sup> to give an intermediate lactone which can undergo direct reduction to the hemiacetal (*cf.* arugosin A/B<sup>3</sup>) and thence to the benzophenone.

In a separate experiment sodium [1-<sup>13</sup>C, <sup>18</sup>O<sub>2</sub>]acetate (90% <sup>18</sup>O) was fed to cultures of *A. varicolor* grown in a normal atmosphere, and the resulting tajixanthone (1) was analysed by <sup>13</sup>C n.m.r. Unfortunately the incorporation level was too low to detect isotope shifts at any carbons except C-13, the C-O bond of which was thereby shown to be acetate-derived.

As expected, shamixanthone (2) isolated in the same experiment with <sup>18</sup>O<sub>2</sub> showed, by mass spectral analysis, the incorporation of three <sup>18</sup>O atoms per molecule. Although the isotope shift in the <sup>13</sup>C n.m.r. of (2) at C-1 could not be completely resolved for accurate determination of the <sup>16</sup>O : <sup>18</sup>O ratio, the presence of <sup>18</sup>O at that site and the reduced <sup>18</sup>O content of the xanthone ring oxygen relative to other sites (Table 1) confirm the operation of the same biosynthetic pathway as that of tajixanthone (1). It is interesting to note that in ravenelin biosynthesis the same type of xanthone ring closure (paths a<sub>2</sub> and b<sub>2</sub>) occurs with retention of the oxygens of a symmetrical dihydroxyphenyl moiety.<sup>9</sup> In contrast, retention of oxygen from the other ring and a single carbon labelling pattern during sterigmatocystin biosynthesis<sup>10</sup> suggest an oxidative coupling mechanism rather than addition-elimination for xanthone formation in that case.<sup>16,17</sup>

We thank the S.E.R.C., N.A.T.O., and the Natural

Sciences and Engineering Research Council of Canada (N.S.E.R.C.) for financial support.

Received, 4th July 1984; Com. 951

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## Lecture Courses

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Lecture courses attended during the period of research include:

"Current Topics in Organic Chemistry" (5 lectures, 1980) by Drs. A.J. Bellamy, I. Gosney, H. McNab, D. Leaver and R.M. Paton.

"Natural Products" (Chemistry Honours course, 5 lectures, 1980-81) by Professor A.I. Scott.

"Biosynthesis" (Environmental Chemistry Honours course, 5 lectures, 1981) by Dr. T.J. Simpson.

"Postgraduate Lectures in Bio-Organic Chemistry" (5 lectures, 1981) by Drs. R.L. Baxter, K. Suckling, P. Jeffs, N. Wilson and W.B. Turner.

"Synthesis and Mechanism of Action of the  $\beta$ -Lactam Antibiotics" (5 lectures, 1982) by Glaxo Group Research.

"Radiochemical Methods" (Environmental Chemistry Honours course, 5 lectures, 1982) by Drs. A.B. MacKenzie and J.E. Whitley (Scottish Universities Research & Reactor Centre, East Kilbride).

"Fourier-Transform Infra-Red Spectroscopy" (5 lectures, 1982) by Dr. S. Cradock.

"The Chemistry of Photographic Processes" (5 lectures, 1983) by Dr. L.A. Williams (Kodak).

"Pulse Sequences and Applications in N.M.R. Spectroscopy" (5 lectures, 1983) by Dr. G.A. Morris.

"1,3-Dipolar Intermediates in Heterocyclic Synthesis"

(5 lectures, 1983) by Drs. J.T. Sharp and R.M. Paton.

"Organic Research Seminars" (3 years attendance) - various speakers.